ENVIRONMENTAL DETECTION AND IDENTIFICATION OF PUTATIVE VIRULENCE DETERMINANTS OF THE ROOT-KNOT NEMATODE (*Meloidogyne* spp.) HYPERPARASITE, *Pasteuria penetrans*

By

LIESBETH M. SCHMIDT

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"Thus was the ending still unfinished,
the finale buried in the future-and in this we find
the fascination of Nature and of Science"
William Beebe – 1921

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LIST OF ABBREVIATIONS

A	Absorbance
BME	β-Mercaptoethanol
ConA	Concanavalin A
ELISA	Enzyme-linked immunosorbant assay
EDTA	Ethylenediamine tetraacetic acid
dH_2O	De-ionized water
DIC	Differential interference contrast
DTT	Dithiothreitol
FPLC	Fast protein liquid chromatography
FISH	Fluorescence *in situ* hybridization
FITC	Fluorescein isothiocyanate
CTAB	Hexadecyl trimethylammonium bromide
IC_{50}	Inhibitory concentration equaling a 50% reduction compared to the control
kDa	Kilodalton
MAb	Monoclonal antibody
GlcNAc	β-1,4-linked N-acetyl-D-glucosamine
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
PBST	10 mM phosphate buffer, pH 7.4, 150 mM NaCl and Tween 20
J2	Second-stage juvenile nematode
SDS	Sodium dodecyl sulfate
TRIS	Tris[hydroxymethyl]aminomethane hydrochloride
TRITC	Tetramethylrhodamine B isothiocyanate
UDC	Urea 6.0 M, CHES buffer 0.005 M pH 9.8, dithiothreitol 0.03 M
WGA	Wheat germ agglutinin

Abstract of Dissertation Presented to the Graduate School
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ENVIRONMETNAL DETECTION AND IDENTIFICATION OF PUTATIVE VIRULENCE DETERMINANTS OF THE ROOT KNOT NEMATODE (*Meloidogyne* spp.) HYPERPARASITE, *Pasteuria penetrans*

By

Liesbeth M. Schmidt

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Major Department: Microbiology and Cell Science

Root-knot nematodes (*Meloidogyne* spp.) are agronomic pests that inflict substantial losses in the worldwide production of a variety of economically important vegetable and other crops. In light of the approaching United States Environmental Protection Agency (USEPA) ban on methyl bromide, a soil fumigant that is currently the most effective nematicide, alternatives are actively being sought. *Pasteuria penetrans* is a Gram-positive endospore-forming bacterium that is an obligate parasite of many plant parasitic nematodes including *Meloidogyne* spp. and is a promising biocontrol agent. *P. penetrans* has not been successfully cultured *in vitro* and therefore cannot be enumerated by traditional bacteriological methods. This has obstructed research on its biocontrol capability. A monoclonal antibody (MAb 2A41D10) produced against *Pasteuria* endospores has been shown to bind an epitope present as a component of the spore envelope. A novel *in situ* spore antigen-extraction procedure is presented that when applied to soil and ground dry plant material is shown to effectively liberate spore envelope antigen for direct application in ELISA. The study analyzed several indirect ELISA schemes to quantify *Pasteuria* spores in soil matrices. Linear regression analysis of soil spore concentrations produced line fits of $r^2 = 0.98$ and indicated a conservative minimum detection limit of ≥ 3000 spores/g of soil. Extraction efficiency was not influenced by soil type for three soils tested. SDS-PAGE immunoblot profiles of soil extracted antigen provided signature peptides upon comparison with the profile of extracts of purified endospores, thus confirming the results of the ELISA. MAb 2A41D10 was challenged with spore envelope extracts obtained from a number of Gram-positive bacteria cultured from test soils and ATTC strains and demonstrated specificity for *Pasteuria* spp. The spore epitope is shown to bind living infective juvenile (J2) nematodes as well as solubilized cuticle peptides. Infective *Meloidogyne* J2 cuticle peptides of M_r~ 34, 45, 55 kDa appear as receptors for spore ligands. Therefore MAb 2A41D10 detects an epitope on spore-borne adhesins that are involved in the attachment of *Pasteuria* endospores to the nematode cuticle as a prerequisite to the infection process. This establishes the MAb as a definitive probe to detect the putative virulence determinant on *Pasteuria* endospores as a basis for estimating soil suppressivity. N terminal sequences were obtained for peptides harboring the MAb epitope and were found to have sequence homology to major histocompatibility complex (MHC) class I molecules and most closely aligned with proteins of the free-living nematode *Caenorhabditis elegans*. This is the first description of a bacterial virulence factor having primary sequence homology to the eukaryotic genome. This suggests a host role in conferring virulence that may be a result of an evolutionary divergence from a symbiotic

or commensal to a parasitic relationship between the bacterium and its host. Using primers designed from recently sequenced *sigE*, *atpF* and *spoIIAB Pasteuria* genes, PCR methods were successfully employed that allowed differentiation of *Pasteuria* infected versus uninfected *Meloidogyne* in crude DNA extracts of root galls. This nucleic acid-based detection system can be used to complement the immunoassay as means to confirm infection in *Meloidogyne* harboring pre-sporulation stage bacterial cellular development.

CHAPTER I
INTRODUCTION

Pasteuria Discovery and Historical Perspective

First described by Metchnikoff in 1888 as a parasite of the Daphnidae *Pasteuria* is a Gram-positive mycelial endospore-forming bacteria (Metchnikoff, 1888). All described members are parasites of invertebrates, specifically freshwater plankton phylum Arthropoda and soil-borne phytopathogenic nematodes of the phylum Nematoda (Ebert et al., 1996). Until recently much confusion and speculation has surrounded its taxonomy including its placement within the Eubacteria. Rühberg (1933) suggested that the cladoceran parasite was a *Torula* yeast, and Jirovec (1936) placed it into the microsporidia, and later in 1939 into the haplosporidian genus *Democystidium* (Ebert et al., 1996). Cobb (1906) was first to report an organism resembling *Pasteuria* spp infecting *Dorylaimus bulbiferus*, a nematode, and suggested it was a sporozoan. This presumption persisted in the literature for some 70 years (Chen and Dickson, 1998). Micoletzky (1925) proposed placing it into the *Duboscqia* and in 1940 a parasite of *Pratylenchus pratensis* was named *Duboscqia penetrans* (Chen and Dickson, 1998). In 1975 Mankau recognized the organisms similarity to spore-forming bacteria rather than to protozoa and named it *Bacillus penetrans* (Chen and Dickson, 1998). Sayre and Wergin (1977) recognized that a parasite of root-knot nematodes (*Meloidogyne* sp.) resembled *Pasteuria ramosa*. Sayre et al., (1983) requested to conserve the name

Pasteuria ramosa Metchnikoff 1888 and the Judicial Commission for the code of nomenclature of bacteria approved it in 1986 (Ebert et al., 1996, Sayre et al., 1983). *Pasteuria*, was "re-discovered" as the bacterial parasite of root-knot nematodes *Meloidogyne* spp. (Sayre and Wergin, 1977) and designated *Pasteuria penetrans* (Sayre and Starr, 1985)

To date four species constitute the genus *Pasteuria*. *Pasteuria ramosa*, (Metchnikoff 1888) the cladoceran parasite, and three species associated with plant parasitic nematodes, *P. penetrans* (Thorne) the *Meloidogyne* spp. parasite, *P. thornei*, the lesion nematode (*Pratylenchus* spp.) parasite (Starr and Sayre, 1988), and *P. nishizawae*, the cyst nematode parasite (*Heterodera* spp. and *Globodera* spp.) (Nishizawa, 1986, Sayre et al., 1991). Distinctive morphological differences in spore shape were observed between the species, which partially result from the shape of the parasporial fibers emanating from the spore core (Sayre and Wergin, 1977). *Pasteuria ramosa* spores are teardrop shaped, *P. penetrans* and *P. thornei* spores are saucer or cup-shaped and rhomboidal shaped, respectively (Chen et al., 1997a, Starr and Sayre, 1988).

Recent 16S rRNA phylogenetic analysis places *Pasteuria* spp. closely with other Gram-positive spore-formers the *Alicyclobacillus* spp., *Thermoactinomyces* spp., *Bacillus* spp., and *Sulfobacillus* spp. (Anderson et al., 1999, Bekal et al., 2001, Ebert et al., 1996)

Control of Root-knot Nematodes (*Meloidogyne* sp.)

Root knot nematodes were first discovered in 1855 by Berkeley on cucumber plants grown in a greenhouse (Christie, 1959). They are sedentary endoparasites residing within root tissues and drawing upon plant nutrient resources to complete their lifecycle.

Adult females reproduce mainly by parthenogenesis, are pyriform in shape and reach sexual maturity within root tissue depositing eggs in gelatinous matrix on the outer surface of the root. *Meloidogyne* males are vermiform in shape and are free-living. Plant infection begins when vermiform second stage juveniles (J2) penetrate plant root systems and migrate to a feeding site. An anterior stylet penetrates the plant vascular parenchyma causing physiological changes to plant cell tissue and the formation of giant cells. Giant cells transfer nutrients to the nematode and away from the plant resulting in decreased growth and productivity. Morphological changes in the root tissue due to prolonged feeding of a nematode inhibit water and nutrient uptake and causes a general reduction in plant health and efficiency of coupling photosynthesis to growth. *Meloidogyne* spp. are estimated to cause crop-losses in the amount of some 80 billion dollars per year worldwide (USDA) and are one of the most widespread and destructive crop pathogens. They pose a significant threat to high value crops such as tobacco and peanut, as well as a variety of economically important vegetables including tomato, pepper, cucumber and eggplant. Figure 1 shows early stages of giant cell or root-gall formation in a tomato plant root system heavily infected with *Meloidogyne arenaria* race 1.

In light of the United States Environmental Protection Agency (USEPA) ban on the use of methyl bromide, which represents the most effective and widely used nematocide (Noling and Becker 1994), alternative methods for control of root-knot nematodes are being actively sought. Chemical alternatives under consideration include chloropicrin, telone, metham sodium, 1,3-dichloropropene, methyl iodide, and Dazomet, many of which have limitations in sandy soils by posing threats to groundwater contamination (Delfino and Miles 1985), or are not entirely effective control agents.

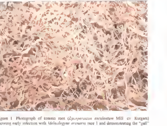


Figure 1 Photograph of tomato root (*Lycopersicon esculentum* Mill cv Rutgers) showing early infection with *Meloidogyne arenaria* race 1 and demonstrating the "gall" formation characteristic of root-knot nematode infection.

Non-chemical alternatives include crop rotation and cover crops, fallow and dry tillage, soil flooding, trap cropping (Christie, 1959, Noe et al., 1991), steam solarization, organic amendments (Rodriquez-Kabana, 1986), and biocontrol (Kerry, 1987, Morgan-Jones and Rodriguez-Kabana, 1987, Stirling, 1991)

Pasteuria penetrans as an obligate parasite of *Meloidogyne* spp has emerged as a promising biocontrol agent for root-knot nematodes (Brown, 1985, Chen and Dickson, 1998, Giblin-Davis, 1990 Gowen and Ahmed, 1990; Tzortzakakis et al, 1997) The promising use of *P penetrans* as a natural biocontrol agent for *Meloidogyne* spp has been widely reported (Bird and Brisbane, 1988, Chen et al., 1996, Eddaoudi and Bourijate, 1998 Oostendorp et al., 1991) (Chen et al., 1997b, Siddiqui and Mahmood, 1999 Stirling, 1984, Tzortzakakis et al, 1997) Five isolates (or biotypes) of *P penetrans* have been described which infect *Meloidogyne* spp in Florida. P20 from *M arenaria* (Neal) Chitwood race 1. Levy County, FL, P25 from *M. arenaria* race 1, Alachua County FL, P100 from *M arenaria* race 2, *M incognita* and *M javanica*, Pasco county FL, P120 from *M incognita* and *M javanica*, Alachua county, FL, and B4 from *Pratylenchus scribneri* Steiner, Seminole County FL which also infects and *M incognita* (Chen and Dickson, 1998 Chen et al, 1997a, Oostendorp et al. 1990) Studies of *Meloidogyne arenaria* race 1 on peanut showed significant suppression of infection and increases in production associated with soil *P penetrans* levels of between 1x10^4 and 1x10^5 endospores/g of soil (Chen et al., 1996). These results mirror similar micro-plot and container studies in which significant reductions in root-knot nematode population densities have been attained through soil application of *P penetrans* spores (Mankau and Prasad, 1975, Oostendorp et al., 1991) Advantages to using *Pasteuria* to control root-knot nematodes include the ability for *in-situ* amplification of the vector in the presence of the host and maintenance of a soil "residual" to address future infection, which are characteristics not exhibited by other potential control measures.

Process of Infection

The initial step in infection is the attachment of the *Pasteuria* spore to the cuticle of second stage *Meloidogyne* juveniles (J2). Germination occurs some 6 to 8 days after the endospore encumbered J2 enters the plant root and begins feeding (Sayre and Starr, 1985). Following infection, by as yet a still unknown mechanism, the female nematode loses fecundity, and the body cavity of the mature female becomes filled with viable endospores. These spores are released into the soil upon death and decay of the nematode and surrounding root tissue (Figure 2). As many as 2 million spores have been observed produced in a single *Meloidogyne* female (Mankau, 1980). In addition to obviating *Meloidogyne* reproduction post-plant infection, endospores can encumber the free-living J2 (at high soil spore densities) to an extent that they are prevented them from invading the plant, which eliminates plant infection and damage entirely (Figure 3).

Entomopathogenic Relatives of *P. penetrans*

Several examples of closely related insect pathogens belonging to the genus *Bacillus* exist, and use of these bacteria for biological control has been widely reported (Aronson et al., 1982, Cheng and Nickerson, 1996, Hofte and Whiteley, 1989, Kalfon et al., 1984, Lecadet et al., 1972, Norris, 1962). *Bacillus sphaericus* produces crystal-like inclusions during sporulation that are toxic to mosquito larvae (Culex sp.) (Kalfon et al., 1984). *Bacillus larvae* is the causative agent for American Foulbrood, a disease affecting honeybee larvae and inflicting losses to the apiary industry. *Bacillus popilliae* and *Bacillus lentimorbus* are related naturally occurring soil bacteria that have been mass produced since the 1940's for control of the Japanese beetle, a turf grass pest. (Hoffmann and Frodsham, 1993). *Bacillus thuringiensis* has undergone extensive development for biocontrol of Lepidopertan, Coleopteran and Dipteran pests. Much is known regarding its virulence machinery, which is expressed primarily in the form of delta-endotoxins by the cry genes during sporulation that occur as crystal inclusion bodies as well as surface localized protein on the spore (Aronson et al., 1982, Cheng et al., 1994, Cheng and Nickerson, 1996, Hofte and Whiteley, 1989, Lecadet et al., 1972). Following ingestion of the spore the crystaline protein is solubilized in the alkaline mid-gut. The solubilized protein undergoes proteolytic cleavage and the "activated" protein diffuses through the peritrophic membrane. The protein binds to gut brush border membranes and causes formation of pores, which disrupt ion gradients, especially potassium dependent amino acid transport. These pores permit germinating spores to migrate into the hemolymph eliciting toxemia followed by septicemia (Cheng and Nickerson, 1996). Several transgenic crops, most notably corn, have been engineered to express one or more varieties of Cry proteins to control numerous economically important pests. Although *P. penetrans* appears to primarily effect fecundity versus having a toxic effect on its host, similarities do exist between *Pasteuria* and the entomopathogenic *Bacillus* spp. Study of the etiology and the molecular basis of virulence in the *Bacillus* spp. may provide insight into some of the unknown mechanisms of germination, sporulation and host preferences within the *Pasteuria*.

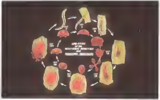


Figure 2 Graphical representation of the concomitant developmental life cycles of *Meloidogyne* sp. and *Pasteuria penetrans* (R.M. Sayre)

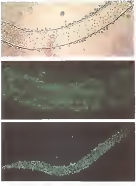


Figure 3 Micrographs showing fluorescein-labeled *P. penetrans* P20 spores attached to the cuticle of *Meloidogyne arenaria* race 1 juveniles (J2). Photographs were obtained with a Nikon phase contrast microscope using at 200X and 400X magnification, and epifluorescence with addition of a 495 nm excitation filter

Pasteuria Spore Coat Epitope and Immuno-detection

Based upon the existing evidence for the presence of a glycoprotein(s) component on the spore envelope (exosporium) as a putative virulence determinant for *Pasteuria penetrans* (Bird et al., 1990, Persidis et al. 1991, Spiegel et al., 1996, Charnecki et al., 1998) this investigation sought to 1) Characterize and utilize this chemical determinant(s) as a tool for detection and enumeration of spores in the environment, and 2) to define specific interactions between the spore (ligand) and nematode cuticle (lectin) which is a basis for virulence. Principal to this investigation is the use of an anti-*Pasteuria* P20 IgM monoclonal antibody (MAb) produced by hybridoma cell line (2A4ID10) in 1997 (Charnecki et al., 1998). This MAb was produced against whole *Pasteuria penetrans* biotype P20 spores in mice. Immunoglobulin M (IgM) and Immunoglobulin G (IgG) are the two most common immunoglobulins in sera or secreted by hybridoma cell lines (Matthew and Reichardt, 1982). IgM is a large ~950 kDa pentameric molecule which consists of five disulfide-linked subunits each containing two heavy and two light chain variable regions (antigen recognition regions) conferring decameric valency (Plaut and Tomasi Jr., 1970). The deca-valency of IgM, provides high substrate avidity (Matthew and Reichardt, 1982).

This research in part sought to prove that MAb 2A41D10 functions as a specific probe for the glycosylated peptide(s) of *Pasteuria spp.* involved in recognition and attachment to the nematode cuticle and can be used to detect viable endospores by binding to an epitope associated with virulence. Existing studies using this MAb have indicated that it is blocked by wheat germ agglutinin (WGA), it inhibits spore attachment to infective *Meloidogyne* J2 by up to ~ 92%, and it has a molar IC_{50} value of 1.3×10^{-10} M

(Charnecki et al , 1998) Specific implications are that this MAb recognizes both portions of the peptide and the glycan by its failure to recognize similar glycan residues present on fetuin, ovalbumin and ribonuclease B (Charnecki 1997)

Research Overview

This work documents the development of a unique *in situ* soil antigen extraction method and a quantitative immunoassay to enumerate *Pasteuria* spores in complex environmental matrices including field soils and plant tissue. Also defined are specific biochemical moieties recognized by the MAb which are associated with spore recognition and attachment to nematode cuticle receptors. Two-dimensional gel electrophoresis and N-terminal sequence analysis of cuticle reactive *Pasteuria* peptides are presented. Additionally the MAb was successfully proteolytically digested with trypsin to generate small immuno-reactive fragments that may be used in portable test kits and for further biochemical evaluation using biosensor technology

The epitope recognized by MAb 2A41D10 appears as a product of sporulation and constitutes a supramolecular structural component of the spore envelope that is not present in vegetative cells. As a result *Pasteuria* in pre-sporulation stages of development, as might be encountered in early infected root systems, would not be detected by immunoassay Conversely, a DNA based PCR approach to detection is not amenable to detection of mature endospores because nuclear material was found to be very poorly released using available DNA extraction methods, which prevents sensitive detection of *Pasteuria* in soil and also in later stages of development within plant systems using PCR. Limitations are inherent to both detection systems and it was necessary to develop a nucleic acid based system to complement the immunoassay

Pasteuria gene specific *SigE*, *SpoIIAB* and *AtpF* nucleic acid primers were developed and applied in real time PCR to crude DNA extracts of infected root tissue that provided for detection of *Pasteuria* vegetative cell development in root-knot nematode infected plants. The presence of spores in soil is not purely indicative of biocontrol potential based upon the complex and as yet loosely defined basis for host specificity between the various *Pasteuria* biotypes and the large number of plant-parasitic nematodes present in the environment. Detection of *Pasteuria* in *Meloidogyne* infected root material using gene specific primers can be used to confirm infection. Due to the greater inherent sequence heterogeneity in these genes as compared to rRNA, *sigE* and *spo* gene sequences from additional *Pasteuria* isolates may be used to generate species and possibly biotype specific primers to differentiate *Pasteuria*. This capability would have a substantial impact on our understanding *Pasteuria* phylogeny and ecology

The significance of determining the biochemical nature of *Pasteuria penetrans* and *Meloidogyne* spp. interaction applies to numerous areas of biology and nematology, both in an applied as well as fundamental manner. At the present time it is not known whether *Pasteuria*, the nematode host, or a combination, confers the epitope(s) associated with *P. penetrans* spore envelope that mediates recognition of and attachment to the *Meloidogyne* host. The presence of distinct host preference(s) and specificity for *Pasteuria* biotypes is suggestive of a host-conferred epitope, or at the least a dynamic genetic host-parasite interaction resulting in variations in virulence. Elucidation of the nature of the determinant may aid in evaluation of the biochemical basis for variation in host specificity between various biotypes, which is of paramount importance in developing further the biocontrol capabilities of this organism. There are potential implications for efforts in in-vitro propagation of *P. penetrans* where more must be learned regarding the biochemical nature of the host-parasite interaction relating to signaling of germination and sporulation in the organism.

From an applied standpoint, the contribution of an accurate and efficient immunoassay to quantify field spore populations and PCR methods to confirm infection will greatly enhance field research, including population dynamics studies and evaluation of *Pasteuria*'s biocontrol capabilities. Research associated with recognizing, analyzing and developing "suppressive soils" with *Pasteuria* will be especially served by this technology.

Elucidation of the chemical determinant holds other promising implications, not the least of which is the potential to formulate target specific "small molecules". Elucidation of the recognition and attachment chemistry could facilitate future development of nematistatic agents, which might function singularly as encumbering agents to prevent root penetration or, as attached to co-effectors, might be used to target biocidal agents directly to the nematode pest. This approach to pest control would be a vast improvement over the use of broad range bio/nematocides. Moreover, these "agents" could potentially occupy the space of bulking agents used in granular fertilizer mixes and be applied economically during crop fertilization or lime application. Along with enhanced solubility cofactors and other such accoutrements to improve activity and dispersion through soil, immediate and non labor intensive control might be obtained. In situations in which it is not feasible to use *P. penetrans* development and use of this technology based on the biochemistry of *P. penetrans* offers a promising alternative.

CHAPTER 2

DEVELOPMENT OF A SOIL ANTIGEN EXTRACTION PROCEDURE AND ENZYME-LINKED IMMUNOSORBANT ASSAY (ELISA) TO DETECT AND QUANTIFY *PASTEURIA PENETRANS*

Introduction

As an obligate parasite of nematodes, *in vitro* cultivation and enumeration of soil spore levels by traditional bacteriological methods is not amenable to the study of *P. penetrans*. Traditionally, soil levels of *Pasteuria spp.* are ascertained using a laborious, time consuming bioassay procedure. Cultivated 2nd stage *Meloidogyne spp.* juveniles (J2) are incubated in a sample soil and isolated by sucrose density centrifugation. The mean number of spores attached to their cuticle is determined by light microscopy and compared to a standard (Chen et al., 1996a). Many of the emerging technologies for detection of bacterial spores in the environment have limited potential for detecting *Pasteuria*, because it is not known what factors stimulate this organism to germinate or to sporulate. Real-time biosensing and nano-detection of bacterial spores rely on the use of specific germinants to elicit spore germination and employ an array of germinogenic enzyme substrates specific for certain enzymes used in traditional bacterial classification (Rotman, 2001). DNA-probing with microarray systems and PCR-based detection are widely utilized for the detection of microorganisms in the environment, but suffer limitations for detection of spore DNA (Fould et al., 2001, Johnson and Tipper, 1981, Vaid and Bishop, 1998). A polyclonal IgY antibody raised against *Pasteuria penetrans* P20 in chickens and recovered by PEG extraction of egg yolks that recognizes *Pasteuria* spore coat epitopes (Chen et al., 1997). This IgY was further evaluated here, and is shown to cross-react with *Bacillus thuringiensis* ssp. *kurstaki* spore coat peptides. Recently, immunofluorescent and ELISA detection using a polyclonal antibody against *P. penetrans* raised in rabbits has been used to detect whole spores in soil following spore recovery by differential sieving (Fould et al., 2001). An effective and efficient means to detect and quantify *Pasteuria* spores is a key component for future evaluation of this agent in biocontrol of plant parasitic nematodes.

This work primarily focused upon development of a unique approach for *in-situ* antigen release and quantification of *Pasteuria* spores in environmental matrices. A monoclonal antibody, (MAb) 2A41D10, produced against *P. penetrans* (P20) spores has been shown to bind a putative glycan epitope shared by peptides of varying mass (antigenic ladder) present as a component of the spore envelope (Charnecki et al., 1998). A procedure is presented for extraction of spore envelope antigen directly from soil or plant tissue under denaturing and reducing conditions. Subsequent application of an enzyme-linked immunosorbant assay (ELISA) of soluble extract using MAb 2A41D10 is used to determine *P. penetrans* spore concentrations in environmental matrices. Unsuccessful attempts to extract spores from soil using immuno-coated magnetic beads is discussed. Specificity of MAb 2A41D10 for *Pasteuria* spp. antigen was evaluated through exposure to a number of both ATCC and Gram-positive soil isolates using SDS-PAGE Western blot, ELISA, and immunofluorescent microscopy. Comparisons of the immunoassay and J2 bioassay were made for a series of test soils originating from peanut plots. The research suggests that *P. penetrans* harbors a unique and specific biochemical moiety present as a component of its spore envelope that can be solubilized in a soil matrix and utilized for immunological detection, limited to the target organism. MAb 2A41D10 was subjected to proteolytic digestion with trypsin, which resulted in production of small immuno-reactive fragments that can be used in future biochemical applications

Materials and Methods

Pasteuria penetrans Cultivation

Pasteuria penetrans isolate P-20 (Oostendorp et al., 1991) originated from *M. arenaria* (Neal) Chitwood race 1, from Levy County, FL, was grown on tomato (*Lycopersicon esculentum* Mill. cv. Rutgers) in green houses. *Pasteuria penetrans* P20 spores were attached to the cuticles of 1 to 5 day-old *Meloidogyne* juveniles (J2) via centrifugation at 4000 RPM for 5 min (Chen et al. 1996a). Infected J2 were transferred via pipet to steam-pasteurized soil in pots containing Rutgers tomato. Inoculations of approximately 10 000 *Pasteuria* infected J2 per week were made for 3 weeks. Isolation of spores followed procedures of Chen et al., (1996a) with slight modifications. Roots were harvested and washed free of debris sixty days after the last inoculation and placed in an 1800 ml beaker containing 10% pectinase (Pemalq, Gist-Brocades), 50 mM NaOAc, and 0.1% $CaCl_2$ at a approximately a 50:50 (v:v) ratio. The roots were digested for 2 days on an orbital shaker table (100 RPM) to free spore-filled female *Meloidogyne* from root galls. Females were collected by decanting onto a 600 µm pore sieve nested on a 150 µm pore sieve under hard water spray. Spore-filled females, identified by a translucent cuticle, were collected by hand using a borosilicate glass pipet with the aid of a dissecting microscope. They were washed with dH_2O, ruptured using a smooth mortar and pestle, and taken through a woven polyester 21 µm filter mesh (Spectra/Mesh) in a 13 mm Swinnex disc holder (Millipore) to remove nematode cuticle and any root debris. Recovered spores were washed in dH_2O at 10 000 x g for 5 min, repeated 2 times, and stored at 4 °C in 0.02% NaN_3 or frozen. Spores were enumerated on a hemocytometer (Fisher) under a 40X DIC objective.

Spore antigen extraction

Spore preparations were added at a ratio of 1.3 (v/v) to a solution with a final concentration of 6 0 M urea, 0 005 M CHES pH 10, and 16 mM dithiothreitol (DTT) to generate pure culture extracts (UDC extracts) with a spore equivalency of roughly 5x10^5 spores/µl (Pandy and Aronson, 1979, Peradis et al , 1991) Soil extractions were performed similarly Soil to be extracted was air-dried and weighed (1 0 g dry weight) into 1 5 ml microfuge tubes and 100 µl of dH_20 (including spores when preparing standards) was added and vortexed To this was added 300 µl of 1.33x stock UDC (DTT added just prior to extraction) followed by vortexing Tubes were incubated for 2 hours at 37 °C or 30 min at 50 °C in a hot water bath with intermittent vortexing and sonic water bath exposure Tubes were then centrifuged 10,000 x g for 1 min and the supernate (UDC extract) removed by pipet. For soils, the supernate was again centrifuged at 10 000 x g for 5 min to remove fines and the supernate transferred to a clean microfuge tube and stored at 4 °C or –20 °C Protein concentrations were determined by Coomassie blue binding assay (Bio-Rad Laboratories) utilizing the method of Bradford (Bradford, 1976)

Immuno-coating of magnetic beads for spore sequestration

MAb 2A41D10 Sephacryl S-300 purified ascites or anti mouse IgM µ-chain specific secondary Ab was bound to Dynal M-450 epoxy coated magnetic beads in an attempt to sequester spores from soil. Beads (4x10^8) were placed in a 1.5 microfuge tube and washed in 1 0 ml of 100 mM sodium phosphate buffer pH 7 7 and re-suspended in

920 µl of same. To this was added MAb containing 200 µg of protein or 300 µg for 2° Ab. The mixture was incubated overnight at 4 °C on an orbital shaker. The supernate was removed by pipet and the beads were suspended in 1.0 ml of PBS (10 mM phosphate buffer, pH 7.4, 150 mM NaCl) containing 1% BSA. This procedure was repeated five times with fresh buffer and then the solution was incubated overnight at 4 °C on an orbital shaker. MAb binding efficiency was checked by comparison of the MAb stock and post-binding supernate using the coomassie blue protein binding assay of Bradford (Bradford, 1976). These results indicated a 22% binding efficiency or 1.13 µg/10^7 beads for the MAb and 22.6% for anti IgM or 3.35 µg/10^7 beads (recommended concentration is 5 µg/10^7 beads). The MAb-coated beads were evaluated by incubation in fluorescein conjugated anti-mouse IgM µ-chain specific Ab and visualized with a Nikon epifluorescent microscope fitted with a Episcopic fluorescence attachment that housed a 495 nm excitation filter to confirm binding of the MAb. A TRITC-conjugated anti-chicken IgY secondary Ab was also used as a negative control.

Spore binding assays were conducted by incubating varying ratios of spores to beads in 1.5 ml microfuge tubes on an orbital shaker.

Soils

Three soils (POL, POD, SHR) were utilized in preparing standards for soil spore extraction. POL is a highly leached quartz fine sand obtained from a Florida flatwoods site. POD was obtained from the same location but was located in a storm water depression and contained visibly more organic matter than POL. SHR is a calcareous loam from a pasture location (Table 1). Soils evaluated for *Pasteuria* spores were collected at IFAS agricultural experimental stations and crop test plots in and around North Central Florida and were obtained from the University of Florida Department of Entomology and Nematology, Gainesville, Florida.

Table 1. Chemical and elemental profile of the soils used to test spore-antigen extraction and prepare standard curves for soil spore quantitation.

Soil	pH	Ca	Mg	K	P	Zn	Mn	Al	Fe	OM
FOL	4.8	17.4	0.6	2.3	1.7	0.57	0.03	170	13.8	0.3
POD	5.1	4.6	0.2	2.9	2	0.71	0.01	407	56.4	0.7
SHR	5.5	323	65.6	54.2	50.7	2.11	27.8	162	31.3	1.1

Elements (mg/Kg)
Organic matter (OM) (weight %)

Antibody production

The cultures producing anti-*Pasteuria* MAb were prepared by the Hybridoma Core Facility, University of Florida (ICBR), Gainesville, FL by immunization of BALB/c mice with 1×10^6 *Pasteuria penetrans* P20 spores. Selection of mass culture 2A41D10 was based upon distinct recognition of spore associated antigens from several different *P. penetrans* biotypes (Charnecki, 1997). Purification of mouse ascites 2A41D10 HL 1325 98-197.5 was achieved by gel filtration on a 1 m Sephacryl S-300 (Pharmacia) column eluted with 0.5 M NaCl and 0.02 M NaPO4 pH 7.0 run at room temperature at a flow rate of 0.6 ml/min, and collected in 6 ml fractions with a Gilson microfractionator. Fractions exhibiting activity against UDC extracts of spores were concentrated using Centriprep–10 microconcentrators (Amicon) and stored in 0.02% NaN_3 at 4 °C.

Polyclonal antibodies were produced in chickens challenged with suspensions of whole spores of *Bacillus subtilis*, *Bacillus thuringiensis*, ssp. *kurstaki* and *P. penetrans*

(P20) in phosphate buffered saline. Antibodies were extracted from egg yolks using the polyethylene glycol (PEG) extraction method (Chen et al , 1997, Polson et al , 1985)

Identification of spore-forming bacteria

Spore forming organisms were isolated and cultured from test soils by heating the soil in sterile dH_2O at 80 °C for 10 min and spreading on TSA plates Single colonies where selected and repeatedly transferred to establish pure cultures. Cultures were sporulated in 125 ml flasks containing nutrient broth (Difco) on an orbital shaker at 100 RPM Gram reactions were recorded on vegetative cells and sporulation was confirmed by endospore staining with malachite green, counter staining with safranin and visualized by phase contrast microscopy Isolates where identified by fatty acid methyl ester analysis (FAME) using a Hewlett-Packard gas chromatograph and the resulting profiles were statistically compared with profiles included in the Microbial Identification System (MIS) (Hewlett Packard) and MIDI Library TSBA version 3 80 (Sasser, 1990a, Sasser 1990b) This work was performed by E. Dickstein, Department of Plant Pathology University of Florida, Gainesville FL.

Enzyme-linked immunosorbant assay (ELISA)

Spore UDC extracts were diluted to a range of between 50 – 0 005 ng/μl protein and used in an indirect standard assay, an amplification assay using biotinylated MAb 2A41D10, and a tertiary ELISA. To 1 μl UDC extract was added 99 μl of coating buffer; (CB 1.59 g Na_2CO_3 , 2.81 g $NaHCO_3$, 0.2 g NaN_3 to 1000 ml dH_2O), and added to a Immulon-2 microtiter plates (Dynatech) Antigen was incubated for 2 hrs at room temperature or overnight at 4 °C, blocked with PBS containing 3% bovine serum albumin (PBS-BSA) for 30 min, washed 3 x with 125 µl of PBS (0.01% Tween 20) pH 7.3 after incubation with each reagent. In the standard indirect ELISA assay plates were treated sequentially for 1 hr with purified anti *Pasteuria* MAb 2A41D10 or mass culture anti-*Pasteuria* MAb 2A41D10 supernatant, and anti mouse IgM (µ-chain specific) alkaline phosphatase-conjugated secondary Ab. For the amplification ELISA, the plates were treated sequentially for 1 hr with biotinylated MAb 2A41D10 (1:1000 PBST 0.1%) and NeutrAvidin™ horseradish peroxidase (HRP) conjugated or alkaline phosphatase conjugated (Pierce). The tertiary ELISA was performed similarly to the standard indirect ELISA with the addition of a biotinylated anti-mouse IgM µ-chain specific secondary Ab (1:500) followed by a tertiary Ab consisting of NeutrAvidin™ horse radish peroxidase (HRP) conjugated or alkaline phosphatase conjugated (Pierce). Bound conjugate was observed by addition of enzyme substrate 3,3',5,5' tetramethylbenzidine, Turbo TMB-ELISA™ (Pierce) and reactions were stopped with 100 µl 1.5 M H_2SO_4 and read at 450 nm on a EIA micro-plate reader (BioRad). Alkaline phosphatase conjugated antibodies were detected by addition of APS buffer containing 0.5 mM $MgCl_2$ and 1.0 mg/ml p-nitrophenyl phosphate and read on a EIA micro-plate reader at 405 nm.

SDS-PAGE and Western blotting

UDC extracts were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Samples were prepared in treatment buffer (50 mM Tris-HCl pH 6.8, 2.0% SDS, 10 % glycerol, and 2% β-mercaptoethanol). A 6% stacking gel and a 12% (wt/vol) separation slab gel was prepared by the method of Laemmli

(Laemmli, 1970). Gels were transferred to nitrocellulose membranes in blotting buffer (192 mM glycine, 25 mM Tris-Base, 20% methanol), using a Mini Trans Blot Cell (BioRad) at 50 V constant voltage for 1.5 hrs. Membranes were blocked with 5% non-fat dried skim milk (Carnation) and 0.2% Tween 20 (v/v) in PBS (10 mM phosphate buffer, pH 7.6, 150 mM NaCl) overnight at 4 °C prior to immunoblotting. Membranes were given three 5 min washes in PBST (10 mM phosphate buffer, pH 7.4, 150 mM NaCl 0.2% Tween 20) and incubated in anti-*Pasteuria* MAb 2A41D10 mass culture supernatant diluted 1:50 or Sephacryl-300 purified ascites diluted 1:100,000, for 1hr washed three times and incubated in anti-mouse IgM μ-chain specific Ab alkaline phosphatase conjugated (Sigma) diluted 1:500 for 1 hr. The blots were given three 5-min washes in PBST followed by three 5-min washes in substrate buffer (100 mM Tris, pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$). Alkaline phosphatase substrate 5 bromo-4-chloro-3-indol phosphate (BCIP) and nitro-blue tetrazolium (NBT) (Promega) were added to the substrate buffer to a concentration of 0.165% and 0.33% (v/v) respectively, for detection of bound MAb. Prestained SDS-PAGE molecular weight markers (BioRad) were run on all gels.

Immunofluorescent microscopy

Circular cover slips (12 mm, #1 thickness), were washed in 70% ethanol and rinsed with excess dH_20. Cover slips were added to a dish containing Alcian blue (cationic dye), 1% in water, and heated to almost boiling for 10 min. The dye was poured off and the cover slips were thoroughly rinsed in dH_20 and dried (light blue in color). Spore suspensions were applied to the cover slips and incubated 1 hr at 30 °C to adhere and dry. Slides were washed 3X in 400 µl PBS pH 7.3 containing 3% bovine serum albumin (BSA). The slides were treated with primary antibody diluted in PBS (3% BSA) for 1 hr, and rinsed 3 times as above. The secondary antibody with a FITC or TRITC-generated fluorescent conjugate was applied for 1 hr, washed 6 times and the slips mounted on ethanol washed standard microscope slides using Fluoromount –G™ (Southern Biotechnology Associates, Inc.). Preparations were stored in the dark and visualized with a Nikon epifluorescent microscope fitted with a Episcopic fluorescence attachment that housed a 495 nm excitation filter under 40X fluorescent, or 100X DIC objectives with oil.

Fluorescent labeling of spores

Spore preparations containing $5x10^7$ spores were centrifuged 10,000 x g for 5 min and reconstituted in 500 µl of a 100 mM bicarbonate: carbonate solution pH 9.1 containing 2.0 mg/ml fluorescein isothiocynate (FITC) (Sigma) (Carraway III et al., 1989; Charnecki, 1997). Spores were incubated 2 hours at room temperature in the dark, centrifuged 10,000 x g for 5 min and reconstituted in dH_20. The process was repeated until the supernatant became clear.

Proteolytic digestion of IgM

Sephacryl S-300 purified MAb 2A41D10 was dialyzed (Mini Slide-A-Lyzer MWC 10 kDa) (Pierce) into trypsin buffer (0.05 M Tris-HCl pH 8.0, 150 mM NaCl, 20 mM $CaCl_2$). Trypsin (Sigma sequencing grade) was added to a final concentration of between 1:50 and 1:1000 (wt/wt) and the samples were incubated in a water bath.

Dithiothreitol was added to a final concentration of 2 mM for 5 min followed by the addition of soybean trypsin inhibitor (Type II-S) (Sigma) to 0.1 mg/ml. After 5 min at 37 °C, iodoacetamide (Sigma) to 60 mM was added and the samples were incubated for 10 min at room temperature. Samples were then dialyzed back into 0.5 M NaCl and 0.02 M sodium phosphate, pH 7.0 and stored at 4°C. Tryptic digests were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 4 – 18% Tris-HCl gradient gels (BioRad) by the method of Laemmli (Laemmli, 1970). Samples were prepared in non-reducing treatment buffer (50 mM Tris-HCl pH 6.8, 2.0% SDS, and 10% glycerol).

Results

Extraction of *Pasteuria* spore antigen from soil

A reagent consisting of 6.0 M urea, 10 mM CHES buffer pH 10, and 3 mM dithiothreitol (UDC), was evaluated for application to release surface antigen from spores derived directly from nematode cadavers and dispersed in soil. Certain conditions were tested to determine requirements and optimum conditions for extraction based on ELISA. Tests varying extraction temperature indicated that elevating temperature to 37 °C or greater (50 °C) was needed to effectively extract spore envelope-associated antigens for the incubation times tested (Figure 4). Varying extraction temperature and time between 37 and 50 °C and 0.5 to 2 hr, respectively, resulted in no significant change in extraction efficiency, thus providing a flexible window with which to liberate antigen. The results of a factorial type combinatorial evaluation of extraction solution components indicated that denaturation with 6.0 M urea was the primary effector of antigen release, followed by alkaline pH conditions provided by 10 mM CHES buffer pH 10 (Figure 5). Interestingly dithiothreitol (DTT), a reducing agent that destabilizes tertiary protein structure by saturating cysteine di-sulfide bonds, had a minor influence on releasing spore antigen (Figure 5). This was additionally evidenced in trials where increasing DTT concentration failed to improve extraction efficiency (Figure 6). This may explain why differing redox and cation-exchange capacities of various test soils did not influence antigen extraction efficiency.

Concerns regarding the potential impacts of the varying physio-chemical properties of soils on spore antigen extraction efficiency and/or signal noise prompted

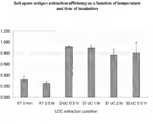


Figure 4 ELISA of 1 µl of POL soil UDC extract containing 4x10^8 spores/g to determine the effect of extraction conditions on the A^{405} nm signal as detected by anti *Pasteuria* MAb 2A4ID10 mass culture supernatant diluted 1:10 in PBST (10 mM phosphate buffer pH 7.4, 150 mM NaCl, and 0.05% Tween 20) and anti-mouse IgM µ-chain specific secondary Ab alkaline phosphatase conjugated. Data shown is an average of six replicates for each treatment.

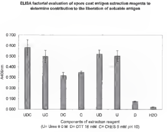


Figure 5 ELISA factorial evaluation of extraction reagents individually and in combination to determine the necessary chemistry required to liberate spore antigen. POL soil (1 g) inoculated with 4x10^6 spores was extracted in 400 µl and 1 µl of extract was added per well Antigen was detected by anti-*Pasteuria* MAb 2A41D10 Sephacryl S-300 purified ascites at a 1 100,000 dilution in PBST (10 mM phosphate buffer, pH 7 4, 150 mM NaCl, and 0 05% Tween 20) and anti-mouse IgM µ-chain specific secondary Ab alkaline phosphatase conjugated. Data represent the triplicate average

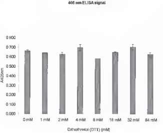


Figure 6. Determination of the impact of dithiothreitol (DTT) on the ELISA A^{405} nm signal. 1 µl of POL soil inoculated with $4x10^6$ spores/g in 400 µl of extract was added per well. Antigen was detected by anti *Pasteuria* MAb 2A41D10 Sephacryl S-300 purified ascites at a 1:100,00 dilution in PBST (10 mM phosphate buffer pH 7.4, 150 mM NaCl, and 0.05% Tween 20) and anti-mouse IgM µ-chain specific secondary Ab alkaline phosphatase conjugated. Data represent the triplicate average

experimentation with more than one type of test soil. Three soils were selected for inoculation and extraction. They represented typical North Central Florida agricultural soils, referred to as POL, POD, and SHR. As is shown in Figure 7, no significant variation in ELISA A 450 nm signal was evidenced for standards prepared in all three soils when evaluated collectively by Box plot analysis (Sigma Plot statistical analysis software). The collective theoretical line fit produced an r^2 of 0.884 over the range of spore concentrations tested.

MAb 2A41D10 sensitivity and specificity

Detection of a relatively high absorbance signal for negative control soil indicated that as much as 50% of background absorbance is due to 2° Ab binding in the absence of 1° for both POL and POD control soils. This indicated that some type and level of blocking agent must be employed in the ELISA procedure in order to mitigate non-antigen specific binding. It was presumed that some indigenous lectin present in the soil extract is binding to the FC (glycosylated) region of the 2° Ab, which was causing the significant background signal. Experimentation using methyl-α-D-mannopyranoside, skim milk and bovine serum albumin (BSA), showed that blocking with 1% or 3% BSA in PBS provided significant reduction in non-specific binding while not compromising overall sensitivity (Figure 8).

Initial tests utilizing the standard indirect ELISA resulted in a minimum detection limit of ≥ 30,000 spores/g (0.056 ug/ul protein) with reproducible r^2 values of > 0.90 (Figure 9). Efforts were made to amplify the signal by biotinylating the MAb to lower

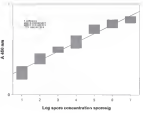


Figure 7 Box plot analysis of the standard indirect ELISA for *Pasteuria penetrans* P20 UDC antigen extracted from inoculated POL, POD, and SHR soils.

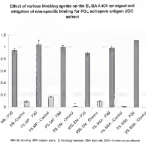


Figure 8: Evaluation of various types and concentrations of blocking agents on mitigation of non-specific binding and ELISA signal strength with respect to antigen concentration for POL soil spore antigen UDC extract

the limit of detection. The MAb was biotinylated using molar ratios of 1:32 and 1:64 of MAb to biotin and detected with NeutrAvidin™ – HRP conjugated and read at A 450 nm. The MAb biotinylated at 1:64 ratio produced a higher absorbance than the 1:34 for a given antigen concentration. However, no increase in sensitivity over the standard indirect assay was observed (Figure 10). The tertiary ELISA provided the best quantitative data and the lowest detection limit. Incubation with MAb 2A41D10, followed by incubation with biotinylated anti-mouse IgM (μ-chain specific) and detected with NeutrAvidin™ – HRP reduced the detection limit over the standard assay at least 10-fold to ≈3,000 spores/g with respectable and reproducible line fits (Figure 11). The minimum detection limit for the ELISA was determined by the lowest concentration within the linear portion of the line fit. Theoretically, the detection limit could be interpreted to approach as little as 30 spores/g; however, low absorbance values relative to sample standard deviations may limit accurate determination of spore concentration at concentrations less than 300 spores/g.

The MAb was challenged with UDC spore extracts of Gram-positive spore-forming organisms isolated and cultured from test soils and American Type Culture Collection (ATTC) strains. Soil isolates were identified using fatty acid methyl ester derivatization (FAME). Figure 12 provides the ELISA results of this challenge. MAb 2A41D10 demonstrated no cross-reactivity with any of the isolates tested.

Reproducibility and precision of the assay

A series of unknown independent samples of dried ground peanut hull and peanut root material containing *Pasteuria penetrans* biotype P100 and P25 spores were weighed

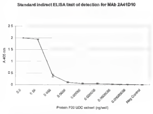


Figure 9 Limit of detection evaluation for the standard ELISA for *Pasteuria penetrans* P20 pure culture UDC extract. 3% BSA (bovine serum albumin) in PBS (10 mM sodium phosphate buffer pH 7.4, 150 mM NaCl) was applied as a blocker for 0.5 hr prior to immuno-detection. Anti-*Pasteuria* MAb 2A41D10 Sephacryl S 300 purified ascites served as the primary Ab and anti-mouse IgM µ-chain specific secondary Ab with alkaline phosphatase conjugate was utilized for detection. Conversion factors: 300 spores/g of soil is equivalent to 0.056 ng/ul protein in the UDC extract. Data represents analysis in triplicate

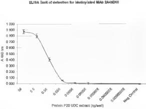


Figure 10: Limit of detection evaluation for the amplification ELISA for *Pasteuria penetrans* P20 pure culture UDC extract. 3% BSA (bovine serum albumin) in PBS (10 mM sodium phosphate buffer, pH 7.4, 150 mM NaCl) was applied as a blocker for 0.5hr prior to immuno-detection. Biotinylated anti-*Pasteuria* MAb 2A41D10 Sephacryl S-300 purified ascites served as the primary Ab and NeutrAvidin™ horseradish peroxidase conjugated secondary Ab was utilized for detection. Conversion factors: 300 spores/g of soil is equivalent to 0.056 ng/ul protein in the UDC extract. Data represents analysis in triplicate

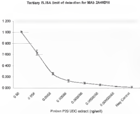


Figure 11 Limit of detection evaluation for tertiary ELISA for *Pasteuria penetrans* P20 pure culture UDC extract. 3% BSA (bovine serum albumin) in PBS (10 mM sodium phosphate buffer, pH 7.4, 150 mM NaCl) was applied as a blocker for 0.5 hr prior to immuno-detection. Anti-*Pasteuria* MAb 2A41D10 Sephacryl S-300 purified ascites served as the primary Ab, anti-mouse biotinylated IgM µ-chain specific secondary Ab and NeutrAvidin^{TM} horseradish peroxidase conjugated tertiary Ab was utilized for detection. Conversion factor: 300 spores/g of soil is equivalent to 0.056 ng/ul protein in the UDC extract. Data represents analysis in triplicate.

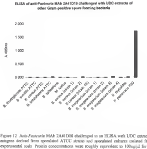


Figure 12. Anti-*Pasteuria* MAb 2A41D10 challenged in an ELISA with UDC extracted antigens derived from sporulated ATCC strains and sporulated cultures isolated from experimental soils. Protein concentrations were roughly equivalent to 100ng/µl for all extracts. Data represents analysis in triplicate

to 200 mg and extracted using a slightly modified soil UDC extraction protocol to account for absorption and rehydration of the material These extracts were then subjected to repeated tertiary ELISA evaluations to test the precision of assay for accessing spore quantity based on a standard curve using successive extractions of POL inoculated soil The results of this evaluation are found in Table 2 Through these tests it was determined that the optimum range in antigen protein concentration for the tertiary ELISA is between 0 005 and 0 5 ng/ul Two types of curves fit the line Log for the 100 X range and linear for the 10 X range. Sample standard deviation for replicate samples is routinely small and even across the range Findings indicate that the assay is reproducible and reliable when assessed through a carefully constructed and properly utilized standard curve

Table 2 Comparison of spore quantification data using 4 sample UDC extracts (EP#1 EP#3, EP#4, and PN) consisting of dried peanut hull and root material showing the precision of the assay Each run represents data generated using freshly prepared standards of POL soil inoculated with *P penetrans* P20

	EP#1	EP#3	EP#4	PN
Run 1	**1.86E+08**	1.64E+09	**BDL**[a]	**1.55E+08**
Run 2	**1.78E+08**	1.80E+09	**BDL**	N/A
Run 3	**1.88E+08**	6.64E+08	**BDL**	**2.11E+08**
Population AVE	**1.84E+08**	1.37E+09	**BDL**	**1.83E+08**
Population STDEV	5.29E+06	6.15E+08	NA[b]	3.96E+07

[a]Below the limit of detection

[b]Not applicable

SDS-PAGE and Western Blot analysis

UDC extracts of cropland test soil and inoculated control soil were compared to *P penetrans* P20 pure culture extracts using SDS-PAGE Western Blot analyses. The presence of *P. penetrans* P20 signature peptides in soil UDC extracts are shown in Figure 13. Peptides with molecular masses (M_r) of 104, 85, 70, 30, 35 kDa and a predominant one at M_r 45 kDa are present in the POL soil inoculum extract. The field soil UDC extract containing native spores as determined by both bioassay and immunoassay exhibits three predominant bands at M_r 85, 70 and 45 kDa.

Immunoassay verses Bioassay

Immunoassay and bioassay quantification of POL soil and soil extracts containing varying known numbers of P20 spores correlated with an r^2 approaching 1.0. Data comparing the two methods on field soil samples obtained from peanut microplot studies are shown in Figure 14.

Immunofluorescent microscopy of MAb binding intact spores

Immunofluorescent microscopy images using fluorescein-conjugated anti-mouse IgM (μ-chain specific) antibody show that MAb 2A41D10 binds to spore coat polypeptides present as both loosely and tightly bound protein on the surface of the endospore (Figure 15a). UDC extracted endospore pellets retain antigen that is seen as a faint halo surrounding the spore (Figure 15c). Mounted sporulated cultures of *B. subtilis* and *B. thuringiensis* ssp. *kurstaki* were unreactive to MAb 2A41D10 (data not shown).

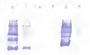


Figure 13. SDS-PAGE Western blot of *P. penetrans* P20 spore UDC extracts and soil P20 spore antigen extracts. The immunoblots were incubated with anti-*Pasteuria* 2A41D10 MAb and anti-mouse IgM μ-chain specific secondary Ab alkaline phosphatase conjugated. Lanes, 2 and 6: UDC solubilized *P. penetrans* P20 spore antigen obtained from culture. 3: UDC extracted POL soil containing 4 x 10^7 *P. penetrans* P20 spores/g, 7: UDC extract of a cropland soil testing positive for 1.2 x 10^6 spores/g of *P. penetrans* spores by ELISA. 4: non *Pasteuria* POL soil (negative control).

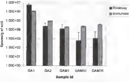


Figure 14: Soil spore levels determined by bioassay and immunoassay ELISA for peanut microplot sample locations at the Institute of Food and Agricultural Sciences (IFAS) Green Acres agricultural station, University of Florida, Gainesville, FL USA

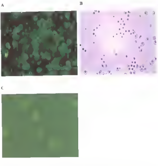


Figure 15. Immunofluorescent micrographs of *Pasteuria penetrans* P20 spores reacting with MAb 2A41D10 and visualized with fluorescein-conjugated anti mouse IgM μ-chain specific secondary Ab. A. differential interference contrast (DIC) 100X objective with oil under epifluorescence with a 495 nm excitation filter. B. same as in A. differential interference contrast (DIC) 100X objective with oil. C. differential interference contrast (DIC) 100X objective with oil under epifluorescence with a 495 nm excitation filter showing immunofluorescence of *Pasteuria penetrans* P20 spores following UDC extraction and retention of epitope bearing UDC insoluble immuno-reactive spore coat proteins.

Immuno-magnetic bead extraction

Attempts to sequester spores from spore suspensions and from inoculated soil using immuno-coated Dynal M-450 beads met with very limited success. Fluorescein-labeled spores that had been treated to repeated sonic water bath and washed bound to the beads (Figure 16), and UDC extracted spores were also shown to bind (Figure 17). MAb binding was observed to fluorescein-conjugated endospore "sluffed" material contained in the supernatents prepared following treatment in a sonic water bath. It appears that labile antigen may compete for whole spore binding to the beads. No spores were recovered from soil following several attempts, using the indirect method (anti-IgM coated beads and pre-incubation of the sample in IgM), or the direct method using IgM coated beads. Soil slurries and soil slurry supernates separated by short pulse centrifugation were both evaluated.

Polyclonal IgY versus IgM

IgM 2A41D10 and an anti-*Pasteuria* polyclonal IgY antibody produced in chickens against *Pasteuria* P20 whole spores provide indistinguishable antigenic ladders in SDS-PAGE immunoblots of UDC extract. However, unlike IgM, the polyclonal Ab recognizes epitopes on other Gram-positive spore-formers and is non-specific for *Pasteuria* sp. The polyclonal Ab reacted positively with several test soil derived isolates in an ELISA. SDS-PAGE immunoblots employing anti-*Bacillus thuringiensis* IgY and anti-*Pasteuria* P20 IgY against UDC extracts of *B. thurigiensis* ssp. *kurstaki* and *P. penetrans* biotype P20 appear in Figure 18. Interestingly, the two Ab's are observed to

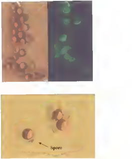


Figure 16. Fluorescein labeled *P. penetrans* P20 spores bound to MAb 2A41D10 coated M-450 Dynal magnetic beads as seen by differential interference contrast (DIC) and epifluorescent microscopy. Panel 1. Complementary DIC and epifluorescent micrograph of spore-bead complex (400X magnification). Panel 2. DIC of spore-bead complex at (400X magnification).

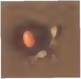


Figure 17. UDC extracted spore pellets of *P. penetrans* P20 spores bound to MAb 2A41D10 coated M-450 Dynal magnetic beads as seen by differential interference contrast (DIC) (900X magnification)

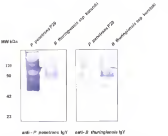

MW kDa
P penetrans P20
B thuringiensis ssp kurstaki
P penetrans P20
B thuringiensis ssp kurstaki
120
90
42
23
anti - P penetrans IgY
anti- B thuringiensis IgY

Figure 18 Antigenic ladders of *P penetrans* P20 and *Bacillus thuringiensis* ssp *kurstaki* spore coat UDC extracts Electrophoretic profiles were produced on 12% Tris-HCl acrylamide gels under reducing conditions and electroblotted onto nitrocellulose membranes. Peptides were detected by anti *P penetrans* and anti *B thuringiensis* IgY polyclonal antibodies produced in chickens

recognize the same epitopes on each respective organism. Predominant shared polypeptides include those with Mr values of 65, 90, and 120 kDa and several minor bands mainly within the 90 to 120 kDa range

Proteolytic digestion of IgM

Trypsin digests of Sephacryl S 300 purified mass tissue culture supernatant produced immunoreactive fragments that could be resolved by both SDS-PAGE and size exclusion FPLC Predominant products had M_r values of 179 kDa and 82 kDa that correspond roughly in size to IgG and Fab^2 fragments, respectively (Figure 19). The protein:trypsin ratio had no apparent effect on the type of cleavage products however incubation time affected product distribution. Incubation at 37 °C for 18 hr was sufficient to convert most IgM into fragments One proteolytic digest of mass tissue culture supernatant produced primarily one fragmentation product having a molecular weight of 83 kDa corresponding to a putative Fab^2 sized fragment (Figure 20a, 20b) FPLC fractions containing this fragment reacted with *Pasteuria* P20 extract upon dot blot analysis (Figure 20d) Boiling of IgM in reducing sample buffer for SDS-PAGE resulted in a similar fragmentation profile as trypsin digestion, which indicates that reducing conditions alone can result in IgM disassembly (Figure 20a).

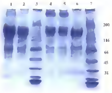


Figure 19 SDS-PAGE 4 15% gradient Tris-HCl acrylamide Coomassie Blue-stained gel of a time course trypsin digest of MAb 2A41D10 (Jan-01) Sephacryl S 300 purified mass culture supernate at 1 100 and 1 500 (protein trypsin) (wt/wt) Samples were boiled for 5 min in non reducing treatment buffer (50mM Tris pH 6 8 2 0% SDS 10% glycerol) prior to loading except were indicated Lane 1 37 °C for 18 hr (1 600) Lane 2 37 °C for 18 hr (1 1000) Lane 3 Molecular mass marker Lane 4 37 °C for 2 hr (1 100) Lane 5 37 °C for 2 hr (1 500) Lane 6 undigested IgM boiled 5 min in reducing treatment buffer (50mM Tris pH 6 8 2 0% SDS 10% glycerol 2 0% β mercaptoethanol) Lane 7 molecular mass marker

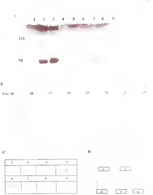


Figure 20 Panel A, SDS-PAGE 4-15% Tris Coomassie Blue-stained gel of IgM 2A41D10 Har#1 mass culture supernatent tryptic digest Lane 1 molecular weight mass markers Lane 2 stock IgM under run reducing conditions Lane 3 Unfractionated IgM trypsin digest. Lanes 4-9 correspond to FPLC fractions 15-20 in Panel B Panel B FPLC size exclusion chromatographic profile of IgM trypsin digest Panel C Dot blot of FPLC fractions recognized by anti mouse IgM μ-chain specific Ab number designations correspond to lane assignments in panel A Panel D Dot blot of *P penetrans* spore UDC extract showing IgM tryptic fractions bind antigen (refer to panel A)

Discussion

Spore antigen extraction from complex matrices

This study introduces an *in situ* soil spore envelope extraction procedure (UDC) which can be employed to solubilize and liberate spore envelope protein from complex matrices for quantitative immunoassay detection either by ELISA or SDS-PAGE immunoblotting. Soil extractions can be performed in less than one hour. This procedure obviates the need to isolate spores prior to immuno-detection.

Our finding that the concentration of the reducing agent (DTT) played a minor role in liberating *Pasteuria* antigen may not be universal for all spore-formers. However, Pandy and Aronson (Pandy and Aronson, 1979) found that increasing DTT concentration did not increase soluble protein in studies on *Bacillus* spores (25-30% of the coat protein remained insoluble). They also found that urea was essential for extraction and coat proteins were most soluble at elevated pH, 10.5 – 11. Urea-β-mercaptoethanol dialyzed extracts of *Pasteuria* spores have been shown to inhibit attachment of spores to *Meloidogyne* juveniles by 60 to 80% (Persidis et al., 1991).

Spore capture using immuno-magnetic beads

Attempts to capture *P. penetrans* spores with antibody coated magnetic beads in soil slurries and soil slurry supernates were unsuccessful. Limited binding, however, was observed for UDC extracted spore pellets and sonic water bath treated fluorescein labeled spores, indicating that MAb orientation on the bead was not a concern. The inability to capture spores in fresh culture supernate was shown to be at least partially linked to the effects of free or labile antigen competing for binding and occupying sites available for whole spores based on observance of labile fluorescein labeled spore material adhering to the beads. Shearing of spore coat material (antigen) by soil particles in the shaking process required to mix the beads may release antigen that inhibits capture of whole spores from soil. The spores are comparatively large with regard to the size of the beads and physical forces may also play a role in their capture from soil. The successful use of magnetic coated beads for sequestration of bacteria from a variety of food and environmental matrices have been widely reported (Bruno and Yu, 1996, Pooler et al., 1997, Pyle et al., 1999; Wipat et al., 1994, Yu and Bruno, 1996), however, difficulties in capture of *Bacillus* spores has been observed (Blake and Weimer, 1997). The successful application of UDC spore antigen extraction procedure obviated the need for further investigation of this approach.

ELISA for detection of *Pasteuria penetrans* in the environment

Quantification of *Pasteuria* spores was demonstrated using three separate ELISA formats. Standard indirect ELISA, amplification ELISA using biotinylated MAb and a tertiary ELISA utilizing a biotin/streptavidin signal amplification system. The biotinylated MAb failed to improve the limit of detection over the standard indirect ELISA but still resulted in a reliable and rapid assay. The tertiary ELISA using a biotinylated anti-mouse antibody followed by NeutrAvidin™ (streptavidin derivative) resulted in the most sensitive assay and was minimally 10-times more sensitive than the standard indirect ELISA. Standard curve generation using POL soil routinely produced line fits of $r^2 \geq 0.90$ for all assays. A conservative minimum detection limit of ≈ 3,000 spores/g for the teriary ELISA falls within the level necessary to detect and evaluate soil nematode suppressivity (Chen et al., 1996b). Blocking with 3% BSA following antigen binding was necessary to mitigate non-specific binding, but it was not necessary to include it in subsequent reaction mixtures.

Detection of signature peptides in SDS-PAGE Western blots of soil extracted antigen obtained from test and field soil qualitatively confirmed the ELISA results and demonstrated definitive evidence that the antigens detected by ELISA were those detected *Pasteuria* on endospores. This observation supports the validity of the assay for quantification of endospores in field samples.

Fragmentation of IgM

Immunoglobulin M (IgM) and Immunoglobulin G (IgG) are the two most common immunoglobulins in sera or secreted by hybridoma cell lines (Matthew and Reichardt, 1982). IgM is a large ~950 kDa pentameric molecule which consists of five disulfide-linked subunits each containing two heavy and two light chain variable regions (antigen recognition regions) conferring decameric valency (Plaut and Tomasi Jr., 1970). This is a large molecule, wherein only a small portion, the Fv region, contains the antigen recognition and binding region. The deca valency of IgM, while being advantageous for substrate avidity, poses limitations for a variety of biochemical applications (Matthew and Reichardt, 1982). IgM can potentially sterically hinder or mask certain biochemical interactions when used to study epitope-binding and receptor inhibition, and is not desirable for surface plasmon resonance or similar biosensor based technology. Responses of IgM to proteolysis vary between IgM molecules (Beale and Van Dort, 1982). Differing responses to proteolysis occur between IgM species due to variation in the amino acid composition of the constant region domains and inter-chain disulfide bridging (Beale and Van Dort, 1982, Lin and Putnam, 1978, Matthew and Reichardt 1982). Factors affecting proteolysis include the type of enzyme employed and the time and temperature of the incubation (Beale and Van Dort, 1982, Lin and Putnam, 1978, Plaut and Tomasi Jr., 1970, Schrohenloher and Bennett, 1971). In few cases does fragmentation proceed in regular steps and produce well defined series of fragmented molecules (Beale and Van Dort, 1982). Proteases commonly employed include trypsin, pepsin and papain. Enzymatic digestion can produce an array of fragments, which can be separated by gel filtration and evaluated for immunological activity by ELISA and characterized by SDS-PAGE and Western Blotting.

This particular IgM lent itself easily and readily to fragmentation with trypsin. We successfully produced immunoreactive IgM fragments that may be used for further research on the P20 epitope using plasmon resonance and other nano- and biosensor-technology. This preparation may be also be important in future marketing of the Ab for distribution in portable test kits.

The P20 epitope

MAb 2A41D10 demonstrated no cross-reactivity with any organisms with which it was challenged in this study. Evidence supports the conclusion that the epitope recognized by this MAb is unique to endospores of *Pasteuria spp.*, at least compared to endospores of other soil-borne bacteria. Immunofluorescent micrographs showed significant antibody recognition of the spore exosporium and following UDC extraction this epitope was still visible as a halo surrounding the periphery of the spore cortex. It appears that this epitope is widely distributed in the inner and outer layers of the spore coat and is a predominant feature. This coincides with the observation of similarities in amino acid composition between insoluble and soluble coat proteins in *Bacillus* (Pandy and Aronson, 1979). In performic acid-oxidized preparations, most tyrosine was absent from hydrolysates of the insoluble fractions, but was replaced by dityrosine residues (Pandy and Aronson, 1979). Dityrosine has been shown to cross-link structural proteins increasing stability.

Previous studies indicate that the epitope recognized by MAb 2A41D10 is involved in recognition and attachment to the nematode, and therefore constitutes a putative virulence determinant (Charnecki, 1997). MAb 2A41D10 has been shown to inhibit attachment of spores to second stage juveniles of *Meloidogyne arenaria* race 1 with an IC_{50} value of 1.3 x 10^{-10} M (Charnecki et al., 1998). Spore attachment to host cuticle is believed to involve lectin-carbohydrate, electrostatic and hydrophobic interactions (Davies et al., 1996; Davies and Redden, 1997; Esnard et al., 1997; Persidis et al., 1991; Spiegel et al., 1996; Stirling et al., 1986). Wheat germ agglutinin (WGA), concanavalin A, N,N'-diacetylchitobiose and periodate inhibit spore attachment to nematodes, implying a β 1 4-linked N-acetylglucosamine containing glycopeptide(s) is responsible for initial recognition of the nematode cuticle. MAb 2A41D10 recognizes UDC solubilized antigen from at least 4 biotypes of *P. penetrans*. Specific implications are that MAb recognizes both portions of the peptide and the glycan by its failure to recognize similar glycans present on fetuin, ovalbumin and ribonuclease B (Charnecki et al., 1998).

The contribution of this sensitive and efficient immunoassay to quantify soil spore populations provides a needed approach to study application of *Pasteuria* for biocontrol of plant parasitic nematodes. Evidence for the specificity of MAb 2A41D10 for *Pasteuria* spp. was demonstrated by the absence of cross-reactivity with soil-borne *Bacillus* spp. and extracts of *Pasteuria* free soils. Comparisons of existing enumeration methods using spore-laden root material show wide variation (Chen et al., 1996a). Of these methods the type amenable to soil quantification (bioassays) are shown to underestimate spore levels (Chen et al., 1996a). The bioassay method also has inherent limitations due to defined host specificities of different *P. penetrans* isolates. Because the immunoassay recognizes virulence-related antigens and correlates well with the J2 bioassay, this methodology should be a valuable predictor of bio-suppressive soils. It has the potential to be used for assessing natural suppressivity of croplands, and could therefore result in reducing application of chemical pesticides that may have detrimental impacts on the environment.

Demonstration here of a procedure to solubilize and detect spore antigen in a soil matrix and in dried ground plant material has potential for broad application in environmental and food microbiology. A rapid screening tool that obviates the requirement of spore isolation prior to detection can greatly reduce processing time and difficulty. Most *in situ* immunocytological detection methods that do not involve the capture and/or isolation of a target organism or its component epitope prior to detection are amenable to quantitation only by direct counting or rely on germination in selective media. This method allows for ease in rapid screening of large sample sets and may be applicable for detection of organisms in a variety of complex media.

CHAPTER 3

ANALYSIS OF HOST AND PARASITE SURFACE COMPONENTS AND INTERACTIONS AS EVIDENCED BY MAb 2A41D10

Introduction

The development of a specific antibody probe for *Pasteuria* and its application for immunological detection and monitoring of spore levels will provide an important tool for assessing the capability of *Pasteuria* spp. as a biocontrol agent of plant-parasitic nematodes. MAb 2A41O10 was further applied as a biomolecular probe to aid in understanding and characterizing the surface chemistry associated with interactions between the *Pasteuria* hyperparasite and its host.

In this study MAb 2A41D10 was evaluated to determine if it recognized a *Pasteuria penetrans* biotype P20 epitope that specifically bound the cuticle of the *Meloidogyne* host. Qualification of the epitope as a virulence determinant would enhance the significance of the MAb as a probe, as it would then likely provide an indication of levels of virulent spores. Based on the resistance, generally, of Gram-positive endospores to environmental insults, it may be assumed that allowing for *Pasteuria*'s requirement to bind to host cuticle to initiate infection, a significant reduction in virulence for *Pasteuria* would result from degradation or deterioration of its spore coat. Detection of this component of the spore may represent the most accurate assessment of soil suppressivity when compared to nucleic acid based detection systems.

The first stage in the host-parasite interaction involves the biochemical interaction of the spore envelope with the cuticle of a second-stage juvenile (J2). The biochemical basis for this interaction is as yet not fully understood. Despite numerous efforts it is, however, thought to primarily involve a lectin-ligand interaction with β-1,4-linked N acetyl-D-glucosamine (GlcNAc) receptor on the surface of the nematode serving as a lectin that recognizes and binds GlcNAc moieties on the surface of the endospore (Bird et al. 1989, Persidis et al., 1991, Spiegel et al., 1996) Four polypeptides have been proposed as potential adhesins (Persidis et al. 1991) When spore inhibitory extract was absorbed onto host cuticle, electrophoresis of the supernatant was depleted of 4 polypeptides (M_r 28,38, 59 and 190 kDa) that appear in the cuticle extract when detected by a polyclonal Ab prepared against whole spores. Electroblotting followed by lectin overlay indicated that all components were glycosylated and contained N-terminal GlcNAc residues. A polypeptide with M_r 47 kDa was also detected with concanavalin A (Con A) suggesting the presence of α-linked glucose or mannose residues. Studies have demonstrated that wheat germ agglutinin (WGA), with specificity for β 1,4-linked GlcNAc residues, Con A, with specificity for α-linked mannose and glucose, N,N'-diacetylchitobiose, or treatment with periodate or endoglycosidase F, inhibit spore attachment to J2, implying a β 1-4-linked N-acetyl glucosamine containing glycopeptide(s) present on the spore coat is likely responsible for initial recognition and attachment. Furthermore, studies on the characterization of nematode cuticle indicate that *Meloidogyne* J2 juveniles have a higher proportion of glycosylated proteins when compared to adults (Reddigari et al., 1986). for which *P. penetrans* endospores have little to no affinity Electrostatic and hydrophobic interactions have also been implicated in attachment (Davies et al., 1996 Davies and Redden, 1997, Davies et al., 1992, Persidis et al., 1991)

The extracuticular components or surface coat of plant parasitic nematodes of the species *Meloidogyne* have been generally described (Davis and Kaplan, 1992, Dickson et al., 1970, Lin and McClure, 1996, Robinson et al., 1989, Spiegel et al., 1997, Spiegel and McClure, 1991, Spiegel and McClure, 1995). Sodium dodecyl sulfate (SDS) extracts of *Meloidogyne javanica* J2 produce a variety of carbohydrate-binding proteins and glycoproteins as evidenced by lectin blotting with WGA and Con A, as well as fucosylated-, mannosylated- and glucosylated-bovine serum albumin (Spiegel et al., 1997). Soybean agglutinin, *Lotus tetragonolobus* agglutinin, and *Limulus polyphemus* agglutinin have also been observed to bind *Meloidogyne* J2 cuticle (Davis and Kaplan, 1992). Western blots of CTAB-solubilized cuticle proteins of infective *Meloidogyne* J2 show differential protein patterns and some variation in Con A binding profiles that may possibly be used for differentiation (Davis and Kaplan, 1992). Differences in carbohydrate content of glycoproteins among different *Meloidogyne* species and races may be related to pathogenicity and host plant specificity (Davis and Kaplan, 1992). A fibronectin-like protein on the surface of J2 root-knot nematodes has been described and it was found that pre-incubation of *Pasteuria* endospores in both heparin and gelatin binding domain fragments of fibronectin blocked adhesion (Mohan et al., 2001). Monoclonal antibodies raised to cuticular epitopes of *M. incognita* J2 resulted in significant reductions in spore attachment to treated nematodes (Esnard et al., 1997).

The principal objective of this investigation was to determine the nature of the epitope recognized by MAb 2A41D10 and whether or not it is involved in recognition and attachment of the spore to the cuticle of the nematode. Two-dimensional gel electrophoresis and N-terminal amino acid sequencing was applied to peptides from *P.*

penetrans P20 endospores recognized by MAb 2A41D10, and nematode surface coat components were identified that bound these peptides in Far Western blots. Protein motif and homologues are discussed for the given spore coat amino acid sequences. Additionally, spores were evaluated following passage through non-preferred host *Meloidogyne* in an effort to reveal the role of the nematode in antigen display

Materials and Methods

Cultivation of juvenile nematodes

Meloidogyne spp. were propagated on tomato (*Lycopersicon esculentum* Mill. cv. Rutgers) in 6 inch clay pots containing steam pasteurized field soil and cultivated in green houses. Following a period of 30 to 90 days plants exhibiting egg masses were harvested and the roots rinsed free of debris with hard-water spray. Egg masses were removed by a 20 sec drench and hand massage in 0.5% sodium hypochlorite (Hussey and Barker, 1973) and collected by decanting through a 75-µm pore sieve stacked over a 25-µm pore sieve. Eggs were thoroughly rinsed to remove residual bleach and placed on Baermann funnels. Juveniles at 2 to 4 days after hatching were collected by decanting the water suspension over a 25-µm pore sieve, rinsed with dH_20 and transferred to a graduated cylinder. The number of J2 were determined by counting the number of individuals in a 1-ml aliquot placed on a counting grid with the aid of a dissecting light microscope. The J2 suspension was pelleted by centrifugation at 1,000 rpm and stored at –20 °C or –70 °C.

Nematode cuticle extraction

Meloidogyne spp. J2 were added at a ratio of (1:3) v/v to a solution with a final concentration of 6.0 M urea, 0.005 M CHES buffer pH 10, and 3 mM dithiothreitol (DTT) in 1.5 ml centrifuge tubes. Samples were incubated at 40 – 45 °C for 1 hr in a hot water bath and subjected to intermittent brief vortexing and immersion in a sonic water bath. After 1 hr the samples were centrifuged at 10,000 g for 5 min and the supernate containing soluble cuticular components was removed by pipet to a fresh tube and stored at 4 °C or –20 °C for longer term storage. Protein concentrations were determined by Coomassie blue binding assay (BioRad Laboratories) (Bradford, 1976). Figure 21 shows the condition of a *Meloidogyne* J2 post UDC extraction and retention of body wall and cavity integrity

SDS-PAGE and Far Western Blotting of nematode cuticle extracts

Cuticle extracts were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 4 – 16% Tris-HCl gradient gels (Bio-Rad) (Laemmli, 1970). Gels were fixed overnight in 50% methanol and 10% acetic acid, washed with dH_2O for 60 min with three changes, soaked in 0.0002% DTT for 30 min, washed in dH_2O for 5 min and incubated with shaking in 0.204% silver nitrate for 2 hrs. Gels were rinsed in dH_2O for 5 min and soaked briefly in 300 ml of solution containing 3.5% sodium carbonate and 0.05% formaldehyde until a homogenous brown color appeared. The solution was poured off and replaced with 500 ml of the same developing solution and the gels were placed on a shaker until bands appeared. The gels were placed in 3% acetic acid for 5 min to stop the reaction, rinsed in dH_2O and soaked in 20% glycerol before drying. To detect nematode cuticle peptides bearing receptors for spore adhesin peptides, a Far Western blotting approach was used in which resolved and blotted cuticle peptides were first probed with spore peptides (UDC extract) and then with MAb 2A41D10. Gels were transferred to nitrocellulose membranes in blotting buffer (192 mM glycine, 25 mM Tris Base, 20% methanol), using a Mini Trans Blot Cell (BioRad) at 50 V constant voltage for 1.5 hrs. Membranes were blocked with 5% non-fat dried skim milk (Carnation) in PBS (10 mM phosphate buffer, pH 7.6, 150 mM NaCl) overnight at 4

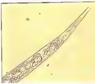


Figure 21 Phase contrast micrograph of a *Meloidogyne arenaria* J2 following urea, CHES and dithiothreitol (UDC) extraction showing intact body wall and retention of body cavity contents

°C before blotting. Membranes were washed in PBST (10 mM phosphate buffer, pH 7.4, 150 mM NaCl 0.2% Tween 20) and blotted with *Pasteuria* biotype P20 UDC spore coat extract (440 ng/µl of protein) at a 1:100 dilution (4.4 mg/ml protein) in PBST for 2.5 hrs shaking at room temperature. The blots were given three 5 min washes in PBST and incubated in anti-*Pasteuria* MAb 2A41D10 mass culture supernatant diluted 1:100 for 1 hr, washed three times and incubated in alkaline phosphatase conjugated anti-mouse IgM µ-chain specific (Sigma) diluted 1:500 for 1 hr. The blots were given three 5 min washes in PBST followed by three 5 min washes in substrate buffer (100 mM Tris, pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$). Alkaline phosphatase substrate 5 bromo-4-chloro-3-indol-phosphate (BCIP) and nitro-blue tetrazolium (NBT) (Promega) were added to the substrate buffer to a concentration of 0.165% and 0.33% (v/v) respectively, for detection of bound MAb.

ELISA of cuticle extract

Extracts containing solubilized surface coat protein from infective J2 were analyzed for protein content by Bradford assay (Bradford, 1976). Extracts were plated at a concentration of 100 ng total protein per well on Immulon-2 microtiter plates (Dynatech) in coating buffer (CB: 1.59 g Na_2CO_3, 2.81 g $NaHCO_3$, 0.2 g NaN_3 to 1000 ml dH_20). Antigen was incubated 2 hr at room temperature or overnight at 4 °C, blocked with PBS containing 3% bovine serum albumin (PBS-BSA) for 30 min, and washed 3 times with 125 µl of PBST (0.01% Tween 20) pH 7.3. Plates were treated sequentially for a minimum of 1 hr with *P. penetrans* P20 UDC extract in PBS (0.02% Tween 20), sephacryl S-300 purified MAb 2A41D10 ascites or mass culture supernatant, and anti mouse IgM (μ-chain specific) alkaline phosphatase-conjugated secondary Ab Plates were washed a minimum of 3 times with PBST (0.01% Tween 20) pH 7.3 between treatments. Bound conjugate was observed by addition of APS buffer containing 0.5 mM $MgCl_2$ and 1.0 mg/ml p-nitrophenyl phosphate on an EIA micro-plate reader at 405 nm (BioRad)

2D-gel electrophoresis of spore coat antigen and *Meloidogyne* J2 surface coat extracts

Pasteuria penetrans P20 endospore coat protein extracted in UDC buffer (4M urea, 10 mM DTT, 0.01 M CHES pH 9.0) was diluted in 150 μl of 9 M urea, 2 M thiourea, 4% CHAPS, 100 mM DTT, 0.001% bromophenol blue and 2% IPG buffer (3-10 NL), the mixture was filtered through a 0.22 μm syringe filter and transferred to a strip holder to rehydrate a 7 cm pH 3-10 (NL) Pharmacia IPG strip for overnight at room temperature Isoelectric focusing (IEF) was conducted using a Pharmacia Multiphor II connected to a Lauda thermocirculator at a temperature setting of 19 °C The focusing condition was set at. 150 V for 30 min, 300 V for 1 hr, 300 V to 3500 V for 1.5 hr and 3500 V for 18.5 hr (analytical) Following IEF the gel strips were either frozen at -20 °C under a layer of mineral oil for later use, or were immediately equilibrated with 2.5 ml of 50 mM Tris-HCl pH 6.8, 6 M urea, 30% (v/v) glycerol 2% (w/v) SDS, 100 mM DTT for 15 min, then with 2.5 ml of 50 mM Tris-HCl pH 6.8, 6 M Urea, 30% (v/v) glycerol, 2% SDS, 2.5% iodoacetamide, and a trace amount of Bromophenol Blue for 15 min. Gel strips were then mounted onto a second dimension Invitrogen Precast 4-20% Tris-Glycine Zoom gel in 0.5% Agarose made in Tris-Glycine SDS electrophoresis running buffer (0.025 M Tris, 0.192 M Glycine, 0.1% SDS, pH 8.3 (Laemmli, 1970)) with trace Bromophenol blue as tracking dye. Novex Mark 12 was used as a mass marker. Gels were subjected to electrophoresis in the running buffer at 125 V for 2 hr. Gels used for silver staining were fixed in 40 % ethanol, 10% acetic acid for at least 2 hr, then silver stained following cross-linking with glutaraldehyde. Gels were transblotted to PVDF membranes at 10 mM MES pH 6.0, 20% methanol at 30 volt for 90 min. For N-terminal sequencing, the transblotted PVDF membrane was rinsed well with dH$_2$O, stained with 0.02% Coomassie Blue R-250 for 30 sec to 1 min, and destained with 40% methanol and 5% acetic acid 2 times for 1 min each. The blot was rinsed extensively with dH$_2$O and air-dried. Western blots and Far Western blots were prepared as for 1-D electrophoresis. Regions of the blot selected for N-terminal sequencing were determined by back to back overlay of the Western blot with the blue-stained sequencing blot. Spots that corresponded with regions of immuno-detection were excised for N-terminal sequencing.

N-terminal sequencing

Edman degradation amino-acid sequencing on excised regions of the Coomassie Blue-stained PDVF membranes was performed using an Applied Biosystems 494 Protein sequencer (P.E. Biosystems) under normal pulse-liquid cycles. Amino acid sequences were made using ABI 610A data software.

Immunofluorescent microscopy of bound spore antigen to *Meloidogyne* J2

Meloidogyne arenaria race 1 juveniles 3 days post hatching (100 J2) were washed in dH$_2$0 and placed in 900 µl of 100 mM MOPS buffer pH 7.0. To this was added 50 µl of *Pasteuria penetrans* biotype P20 UDC spore coat extract containing 10.2 µg total protein. The negative control consisted of 50 µl of J2 in 950 µl MOPS buffer. Samples were placed in microfuge tubes and incubated on a rotary shaker for 1 hr. They were washed three times by 30 sec pulse centrifugation in PBS (10 mM phosphate buffer pH 7.3, 300 mM NaCl). Anti-*Pasteuria* MAb 2A41D10 purified from ascites on sephacryl S-300 at a 1:1000 dilution in PBST (0.2% Tween) pH 7.3 was added to the samples, followed by incubation for 1 hr on a rotary shaker. The previous wash step was repeated and the samples were then incubated in anti-mouse IgM µ-chain specific secondary antibody labeled with fluorescein (Sigma) for 1 hr with shaking. The samples were washed four times as described above and slide mounts were prepared by placing the specimens on glass sides with cover slips. Immunoflourescent microscopy was performed using a Nikon epiflouresence microscope housing a 495 nm excitation filter.

FPLC of *Pasteuria* P20 and *M. arenaria* J2 UDC extracts

Fast protein liquid chromatography (FPLC) of UDC extracts was performed using a Biologic FPLC (BioRad) equipped with a Bio-Sil SEC-125 column and eluted in 100 mM $NaPO_4$ pH 7.6 and 150 mM NaCl at a flow rate of 1 ml/min.

Differential attachment and propagation of *Pasteuria* biotypes

Egg masses from populations of *M. incognita* race 1, *M. incognita* race 3, *M. arenaria* race 1, *M. arenaria* race 2 and *M. javanica* race 1 were propagated and extracted as stated previously. Baermann funnels were harvested at 1 to 5 day intervals for infective J2. *P. penetrans* biotype P20, P100, P120 and B4 spore suspensions were added to 15 ml tubes containing J2. Tubes were centrifuged at 1000 rpm for 5 to15 min or until 100% attachment was attained. In some cases attachment was difficult and more spores were added and/or centrifugation times were extended until at least 60% of J2 had at least one spore attached.

Results

Immunofluorescent micrographs of cuticle bound antigen

Immunofluorescent micrographs of Meloidogyne *arenaria* race 1 J2 pre-incubated in *Pasteuria* P20 spore UDC extract and exposed to MAb 2A41D10 show the epitope recognized by the MAb binds to the cuticle of infective J2 (Figure 22). The results show the entire surface of the nematode contains receptors for spore coat protein. *Pasteuria* spore extract is shown to bind only to *Meloidogyne* J2 and not males or free-living nematodes, i.e. *Rhabditis* spp. (Figures 23 and 24)

SDS-PAGE and Far Western blotting

SDS-PAGE and Far Western blots of resolved UDC-solubilized *Meloidogyne arenaria* race 1 and *Meloidogyne incognita* race 3 J2 surface coat protein reveals similar profiles containing several molecular weight species. Predominant bands at M_r ~34, 45 and 55 kDa appear on both blots. The band at M_r ~34 kDa is not present in the electrophoretic profile of *P. penetrans* P20 (positive control), thus providing additional proof of this as a nematode derived protein and not a result of contamination. (Figure 25) MAb 2A41D10 showed no cross-reactivity with cuticle extract in the absence of pre-incubation with spore extract and produced a blank lane on the blot (data not shown). The corresponding silver stained gel shows a generally unresolved continuum of product(s) and fails to demarcate the reactive peptides seen in the Far Western blot, suggesting that these peptides either do not stain well or that the component binding the putative adhesins are present as a minor component of the nematode surface coat.

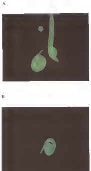


Figure 22 Panel A and B. Infective *Meloidogyne arenaria* race 1 J2 pre-incubated in *P. penetrans* isotype P20 UDC solubilized spore envelope protein and detected by MAb 2A41D10 and anti mouse IgM μ-chain specific fluorescein isothiocyanate (FITC) conjugated secondary Ab under epifluorescence using a 495 nm excitation filter

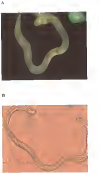


Figure 23 *Meloidogyne arenaria* race 1 male pre incubated in *P. penetrans* biotype P20 UDC solubilized spore envelope protein and exposed MAb 2A41D10 and anti-mouse IgM µ-chain specific fluorescein isothiocyanate (FITC) conjugated secondary Ab. Panel A. as viewed under epifluorescence using a 495 nm excitation filter; Panel B. under brightfield microscopy

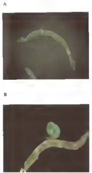


Figure 24 Panel A *Rhabditis* sp free-living nematode pre-incubated in *P penetrans* biotype P20 UDC solubilized spore envelope protein and exposed MAb 2A41D10 and anti mouse IgM μ-chain specific fluorescein isothiocyanate (FITC) conjugated secondary Ab as viewed under epifluorescence using a 495 nm excitation filter Panel B Comparison of non-immunofluorescent control with infective *Meloidogyne arenaria* race 1 J2 (top) Note the presence of internal auto-influorescence which illuminates a yellow color under excitation at 495 nm as compared to FITC (green)

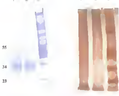


Figure 25 Far Western blot and silver-stained gel of UDC cuticle extracts of 1 to 7 day old *M arenaria* race 1, *M. incognita* race 3 J2 and *P penetrans* P20 UDC extracted spore envelope protein 4-15% Tris-HCl gels were used for SDS-PAGE Proteins for the Far Western blot were detected by incubation in *Pasteuria* P20 UDC extract, followed by incubation in MAb 2A41D10 and anti mouse IgM μ-chain specific Ab alkaline phosphatase conjugated Lanes 1 and 2 are Far Western blots of electrophoresed cuticle extracts and Lane 3 is a Western blot of *Pasteuria penetrans* spore coat extract. Lane 1 *M arenaria* race 1 Lane 2, *M. incognita* race 3, Lane 3, *P penetrans* P20 Lanes 4-6 Silver-stained gel as in Lanes 1-3

ELISA of spore antigen binding

Some blocking reagents commonly used in ELISA reduced P20 antigen binding. It was found that 3% skim milk (Carnation) reduced the A 405 nm signal by 23% and 3% gelatin (bovine skin type A) reduced the signal by 16% when compared to 3% bovine serum albumin (BSA) which reduced the signal just 2% over the non-blocked control (Figure 26). The nature of the blocking agent had a significant effect on the ability to detect binding of spore coat antigen to cuticle. UDC solubilized infective J2 cuticle extracts plated at 100 ng/ well were challenged with *P. penetrans* P20 extract ranging from 5 to 250 ng to determine if the epitope recognized by MAb 2A41D10 bound cuticle in a dose dependent manner. Bound antigen was detected using anti-*Pasteuria* MAb 2A41D10. Spore antigen is shown to bind cuticle extracts of *M. arenaria* race 1 and *M. incognita* race 3 in dose-dependent fashion. *M. incognita* demonstrated higher absorbances than *M. arenaria* for a particular spore extract concentration (Figure 27)

2-D gel profiles and N-terminal sequence of *Pasteuria* immuno-reactive peptides

Pasteuria penetrans P20 UDC extracted spore envelope proteins where separated by 2-dimensional gel electrophoresis (Figure 28). N-terminal amino acid sequencing by Edman degradation was performed on resolved MAb 2A41D10 reactive peptides (Figure 29). Spots corresponding to peptides of M_r~ 23, 96, and 120 kDa were partially sequenced. Two peptides having a M_r of 96 kDa had different isoelectric points (Figure 29 spots 1 and 3) and different N-terminal amino acid sequences. Three of the four peptides began with a serine residue and two contained a glutamic acid at the second position (Table 3). An isoform of a 23 kDa peptide (Figure 28 and 29 spot 4) provided

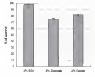


Figure 26: ELISA of *M. arenaria Race* 1 J2 cuticle extract and the effect of various blocking reagents on detection by P20 spore extract using anti-*Pasteuria* MAb 2A41D10

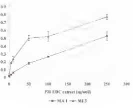


Figure 27 Dose response binding of *Pasteuria* P20 spore coat extract to *M. arenaria* race 1 and *M. incognita* race 3 cuticle extracts as detected by anti-*Pasteuria* MAb 2A41D10 in an ELISA. Cuticle extracts were plated at 100 ng/well

Figure 28 2-Dimensional gold-stain blot of *Pasteuria penetrans* P20 spore envelope proteins IEF pH 3-10 SDS PAGE on 12.5% Tris HCl

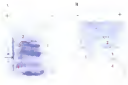


Figure 29 2-dimensional gel blots showing cut sites for N-terminal amino acid sequencing of *Pasteuria penetrans* P20 MAb 2A41D10 spore envelope immuno-reactive peptides Panel A Coomassie Blue-stained sequencing blot The sites indicated 1 through 4 in red were used for individual sequence analysis The arrow indicates a miscut that was not sequenced Panel B Mirror image (reverse side of the gel used for Panel A) of the Western blot that was used to determine cut sites The two blots were overlayed (back to back) and a sterile needle was used to perforate areas on the sequencing gel that corresponded to immuno-reactive regions on the Western blot the longest congruent amino acid sequence

Table 3 N-terminal amino acid sequences for resolved 2-dimensional gel electrophoresis (Figure 28) *Pasteuria penetrans* P20 spore coat proteins bearing the epitope recognized by MAb 2A41D10

Polypeptide[a]	MW (kDa)	N terminal sequence
1	96	MYHNDYQGKX[b]
2	120	S/G/A[c] F L/G/Q XX
3	96	SEGNIXGGXRV X/L X/E A
4	23	S/G EE Q/G XXYKPPAML P/F QR

[a] Refer to 2-dimensional gel for identifications in Figure 28

[b] X, Modified amino acid - unable to assign an identity

[c] / Amino acids listed in decreasing order of likelihood for a particular position

Sequence analysis of MAb reactive spore envelope proteins

N-terminal amino acid sequences were obtained for four *P. penetrans* P20 UDC solubilized peptides that contained the epitope recognized by MAb 2A41D10. Generalized motif expressions as provided by Peptool software (Biotools) are presented below in Table 4. In terms of homology, two of the three sequences aligned most significantly with *Caenorhabditis elegans* proteins using NCBI BLAST parser search. *C. elegans* hypothetical protein Y43F4B.2 (accession no. T26840) had the most significant similarity to spot 1 (96a kDa). Spot 3 (96b kDa) aligned most significantly to a hypothetical protein MLCB2533.09 of *Mycobacterium leprae* (accession no. CAA22923). But it also showed some similarity to *C. elegans* hypothetical protein BO331.1 (accession no. T18699) among other eukaryotic MHC Class I type proteins. Spot 4 (23 kDa) had primary sequence homology to a 26.5 kDa hypothetical protein from *C. elegans* (accession no. Q09652) and *C. elegans* hypothetical protein FA2A10.5

(accession no. T30959) Surprisingly, none these sequences exhibited homology to other bacterial spore coat proteins Interestingly, only one of these three peptides showed significant similarity to other eubacteria. The similarity of these peptides to the free-living nematode *C. elegans* and with peptide motifs similar to immune system proteins, MHC Class I and a generalized B cell epitope, can not be easily explained. It suggests the possibility that the presence of these genes may have arisen as a result of development within a eukaryotic host.

Table 4 Motif assignments for MAb 2A41D10 reactive peptides of *P. penetrans* spore envelope proteins as shown in Figure 28 using Peptool (Biotools software). References for the assignments were provided by Peptool.

Polypeptide	MW (kDa)	Sequence	Motif
1	96	MYHNDYQGKX ***********	Generalized B Cell epitope[1]
3	96	SEGNIXGGXRVXXA ************	MHC Class I epitope[2]
4	23	SEEQXXYKPPAMLPQR ***********	MHC Class I epitope[3]
4	23	SEEQXXYKPPAMLPQR *******	Glycogen synthase kinase 3 phosphorylation site[4]

[1] Wishart, D S (unpublished)
[2] Rammensee, H-G et al., Immunogenetics 41:178-228
[3] Falk, K. et al , Nature 351:290-296
[4] Fiol, C.J et al., J. Biol Chem 262 14042-14048

Biochemical analysis and profiles of *Pasteuria* and *Meloidogyne* J2 UDC extracts

Fast protein liquid chromatography (FPLC) profiles of *P. penetrans* spore envelope UDC extracts and *M. arenaria* race 1 surface coat UDC extracts separated by size exclusion, provided a large number of peptides. Dot blots of individual fractions of spore UDC extract where tested for recognition by MAb 2A41D10 The results indicate the epitope is distributed among the entire chromatographic range, as all fractions are shown to be recognized by MAb 2A41D10 (Figure 30).

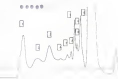
The FPLC profile of cuticle extract shows a majority of the UDC-solubilized cuticle protein eluted as relatively low molecular weight peptides based on absorbance at 280 nm. Large peaks at M_r 18.8, 15.3, 21.1 and 8.9 kDa are seen to dominate the profile (Figure 31). Two-dimensional gel electrophoretic profiles of *Meloidogyne arenaria* J2 UDC solubilized cuticle extract are shown in Figure 32. In an attempt to characterize the cuticle receptor for the spore coat ligands N-terminal amino acid sequencing was performed on three excised peptides that bound *Pasteuria penetrans* P20 spore coat extract as indicated by reactivity to a Far Western blot. These peptides unfortunately did not provide sequence information and appeared to be N-terminally blocked.

Differential attachment and propagation of *Pasteuria* biotypes

In this study *P. penetrans* biotypes P20, P100, P120 and B4 were forcibly attached to a variety of preferred and non-preferred host *Meloidogyne* spp. and propagated. The spore progeny were evaluated for changes in peptide size and distribution following development in different hosts. Table 6 provides biotype designations and host preferences for *Pasteuria* spp. Individual biotypes of *P. penetrans* display characteristic antigenic ladders upon SDS-PAGE Western blot analysis using MAb 2A41D10. Some molecular mass peptides are commonly shared between individual biotypes but vary in distribution and some are biotype specific. Western blots obtained with MAb 2A41D10 for UDC extracts are given in Figures 33-36. The results overall indicate that peptide mass distributions are maintained following a single passage on a non-preferred host. In figure 35 lane 5, in which P120 was propagated on *M. arenaria* race 2, spore attachment was difficult and subsequent infection was poor. As a result very few spores where produced which is the basis for the weak signal on the blot. Biotype P20 (Figure 33), the only isolate for which there is only one preferred host *(M. arenaria* race 1) demonstrated a slightly altered profile. A peptide of $M_r \sim 84$ kDa is absent in lane 5, and in lanes 4 and 6 a $M_r \sim 64$ kDa peptide is not represented as compared to the control (Lane 2)

Table 6. *Pasteuria penetrans* biotype designation, host preference and geographical location where first isolated

Biotype	Host preference	Geographical location
P20	***M. are 1***	**Levy Co., FL, USA**
P25	***M. are 1***	**Alachua Co., FL, USA**
P100	***M. are 2; M. inc; M. jav***	**Pasco Co., FL, USA**
P120	***M. jav; M. inc***	**Alachua Co., FL, USA**
B4	***P. scribneri, M. inc***	**Seminole Co., FL, USA**



Peak No.	Fraction No.	MW (kDa)
1	15	654
2	17-18	81.4
3	20-21	18.5
4	22-23	5.1
5	24	2.5
6	25	1.7
7	25-26	<1.35
8	26	<1.35
9	27	<1.35
10	27	<1.35

Figure 30. FPLC profile and fraction collection of *P. penetrans* P20 UDC spore envelope extract separated by size exclusion chromatography and detected by UV absorbance at 280 nm. Molecular weight designations for peaks 1 through 10 were calculated by linear regression using retention times of known protein standards. The large peak at the end of the run is a urea solvent peak. The dots appearing across the top of the chromatogram are dot blots using 10 µl of each fraction showing immuno-reactivity with MAb 2A41D10.

Peak No	MW (kDa)
1	> 670
2	267.6
3	101.3
4	58.8
5	18.8
6	17.4
7	15.3
8	12.1
9	8.9
10	<1.35

Figure 31. FPLC profile of UDC-solubilized surface [illegible] from [illegible]gyne *arenaria* race 1 infective J2. Peptides were separated by size-ex[illegible] chromatography and detected by UV absorbance at 280 nm. Molecular weight designations for peaks 1-10 were calculated by linear regression using retention times of known protein standards.

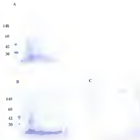


Figure 32 2-dimensional gel blots used for N-terminal amino acid sequencing of *Meloidogyne arenaria* race 1 UDC extracted surface coat peptides that bind *Pasteuria penetrans* P20 spore envelope peptides that are recognized by MAb 2A41D10 Panel A Coomassie Blue stained blot Panel B Coomassie Blue-stained blot with cutouts excised for N-terminal sequence amino acid analysis. Panel C Far Western blot of cuticle extracts blotted with *Pasteuria* P20 spore extract and detected with MAb 2A41D10

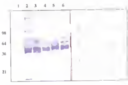


Figure 33 Antigenic ladder of *Pasteuria penetrans* biotype P20 propagated on several host and non-host *Meloidogyne spp* as detected by MAb 2A41D10 Lane 1 Molecular weight markers (kDa) Lane 2 *P penetrans* P20 stock propagated on *Meloidogyne arenaria* race 1 (host) Lane 3 *P penetrans* P20 propagated on *Meloidogyne javanica* race 1 (non host) Lane 4 *P penetrans* P20 propagated on *Meloidogyne incognita* race 1 (non-host) Lane 5 *P penetrans* P20 propagated on *Meloidogyne arenaria* race 2 (non-host) Lane 6 *P penetrans* P20 propagated on *Meloidogyne arenaria* race 1 (host) Right, densitometer scans of lanes 2-6 (top to bottom)

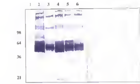


Figure 34. Antigenic ladder of *Pasteuria penetrans* biotype P100 propagated on several host and non host *Meloidogyne* spp. as detected by MAb 2A41D10. Lane 1. Molecular weight markers (kDa). Lane 2, *P. penetrans* P100 propagated on *Meloidogyne arenaria* race 1 (non-host). Lane 3. *P. penetrans* P100 propagated on *Meloidogyne arenaria* race 2 (host). Lane 4. *P. penetrans* P100 propagated on *Meloidogyne incognita* race 1 (host). Lane 5. *P. penetrans* P100 propagated on *Meloidogyne javanica* race 1 (host). Lane 6. *P. penetrans* P100 propagated on *Meloidogyne* spp. laboratory stock (host). Right: densitometer scans of lanes 2-6 (top to bottom).

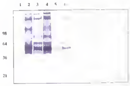


Figure 35 Antigenic ladder of *Pasteuria penetrans* biotype P120 propagated on [illegible] host and non host *Meloidogyne* spp as detected by MAb 2A41D10 Lane 1 Molecular weight markers (kDa) Lane 2 *P penetrans* P20 propagated on *Meloidogyne arenaria* race 1 (host) Lane 3 *P penetrans* P120 propagated on *Meloidogyne javanica* race 1 (host) Lane 4 *P penetrans* P120 propagated on *Meloidogyne incognita* race 1 (host) Lane 5 *P penetrans* P120 propagated on *Meloidogyne arenaria* race 2 (non host) Lane 6 *P penetrans* P120 propagated on *Meloidogyne arenaria* race 1 (non host) Right, densiometer scans of lanes 2-6 (top to bottom)

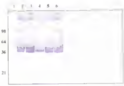


Figure 36. Antigenic ladder of *Pasteuria penetrans* biotype B4 propagated on several host and non-host *Meloidogyne* spp. as detected by MAb 2A41D10. Lane 1. Molecular weight markers (kDa). Lane 2. *P. penetrans* P20 propagated on *Meloidogyne arenaria* race 1 (host). Lane 3. *P. penetrans* B4 propagated on *Meloidogyne javanica* race 1 (non-host). Lane 4. *P. penetrans* B4 propagated on *Meloidogyne incognita* race 1 (host). Lane 5. *P. penetrans* B4 propagated on *Meloidogyne arenaria* race 2 (non host). Lane 6. *P. penetrans* B4 propagated on *Meloidogyne arenaria* race 1 (non host). Right, densitometer scans of lanes 2-6 (top to bottom).

Discussion

This study provides definitive proof that a *Pasteuria penetrans* endospore coat epitope recognized by MAb 2A41D10 binds to the cuticle of infective *Meloidogyne* J2. This finding further supports a role for this epitope as a virulence determinant. This in turn defines the value of the immunoassay as a means to detect virulent spores by recognizing peptides involved in recognition and attachment to the host. This finding has strongly implicated specific peptides both on the spore and the nematode that are required for recognition and attachment. The interaction between spore (glyco)peptides and cuticle receptors, a putative lectin-ligand interaction, is remarkably maintained under reducing and denaturing conditions. Released spore coat protein is shown to bind both to live J2 as shown by immunofluorescent microscopy, and solubilized surface coat extracts in Far Western blots. This interaction is the basis for further characterization of cuticle receptors

MAb 2A41D10 recognizes ligands of cuticle receptors

Immunofluorescent microscopy revealed that the epitope recognized by MAb 2A41D10 present in soluble spore envelope extract of *Pasteuria penetrans* biotype P20 binds *Meloidogyne arenaria* race 1 J2 cuticle. Spore ligand receptors on the nematode are located over the entire cuticle surface and are not restricted to specific regions such as excretory organs, amphids and phasmids (Page et al. 1992). This is consistent with observations on spore attachment as endospores are observed to attach at sites uniformly distributed over the nematode surface. Spore coat protein extract did not bind to non-host *Meloidogyne* males or free-living nematodes, indicating the occurrence of a specific interaction. The nematode surface coat is known to exhibit stage-specific ultra-structural and compositional differences (Blaxter et al 1992 Persidis et al 1991, Phillip et al., 1980; Reddigari et al., 1986) (Rudin 1990) Differences in soluble cuticle fractions between adult *Meloidogyne* females and juveniles notably appear in the prevalence of glycoproteins in juveniles verses their absence in adult females, for which *Pasteuria* has no affinity (Reddigari et al., 1986) A variety of lectins bind to the soluble body wall proteins of infective *Meloidogyne* juveniles (Davis and Kaplan, 1992)

Far Western blots of resolved infective *Meloidogyne arenaria* race 1 and *Meloidogyne incognita* race 3 J2 surface proteins released by urea, CHES and dithiothreitol extraction buffer were probed with *P penetrans* spore coat extract and visualized by anti-*Pasteuria* MAb 2A41D10 The results revealed several molecular weight species as cuticle receptors. Polypeptides of approximate M_r 34, 45 and 55 kDa resolved as strong and distinct bands The antigenic ladder included several fainter bands and fell within a range of between approximately 25 kDa and 60 kDa. MAb 2A41D10 showed no cross-reactivity with cuticle extract in the absence of pre incubation with spore extract. ELISA substantiated the qualitative results of the interaction, and demonstrated that *P penetrans* spore extract bound immobilized cuticle extract in a dose dependent manner

Davies (1994) described a putative cuticle receptor having a M_r~190 kDa. However the polyclonal antibody to *P penetrans* used in the study cross-reacted with other epitopes in the cuticle extract (Davies, 1994) In a recent study, antibodies to human fibronectin were shown to bind a series of polypeptides of M_r 40 45 and 55 kDa present in cuticle extracts of *M javanica* J2. Pre-incubation of *Pasteuria* spores with the heparin and gelatin-binding domains of human fibronectin inhibited attachment to host cuticle, thus suggesting a possible chemistry for attachment (Mohan et al., 2001). We found that bovine gelatin reduced recognition of cuticle by spore extract in ELISA by 16%. Mohan et al., (2001) reported a reduction in attachment of 20% when J2 were pre-treated with 1% gelatin, which was consistent with our findings (Mohan et al., 2001). However, pre-incubation in skim milk also reduced recognition by 23%. The peptides on the nematode cuticle that bound human anti-fibronectin antibodies are interestingly the same relative size to those bound by *P. penetrans* UDC extract in Far Western blots in this study. This suggests that a possible fibronectin-like lectin is recognized by *P. penetrans* spore coat peptides and these adhesins contain an epitope recognized by MAb 2A41D10.

Unfortunately, attempts to N-terminal sequence the cuticle receptors for evidence of a fibronectin like motif or sequence homology were unsuccessful. Indications are that the N-termini of the polypeptides were blocked, thus preventing Edman degradation. Edman degradation requires a free amine group on the N terminus of the peptide where phenylisothiocyanate (PITC) reacts under mildly alkaline conditions to form a phenylthiocarbamyl adduct. Subsequent treatment with a strong acid (trifluoroacetic acid) hydrolyzes the C-N bond between the first and second amino acid leaving a second amino acid free as an H_3N^+ and the N-terminal residue as a thiazolinone derivative. This derivative is extracted into an organic solvent and stabilized in mild acid and analyzed by comparison with known standards. If the N-terminal amine is blocked, then derivatization and Edman degradation cannot proceed. FPLC chromatographs and silver stained gels of UDC extracted nematode surface coat indicate that the putative receptor is present in very low quantity. Trypsin digestion to create free internal amino groups was considered, but based on the small amount of protein which comprised the reactive portion of the total extract, it would be unlikely that enough protein could be recovered to undergo analysis.

N-terminal sequence homology for MAb 2A41D10 spore envelope epitopes

Pasteuria spore coat adhesins demonstrated closest sequence homology to eukaryotic MHC Class I proteins notably belonging to *Caenorhabditis elegans*. Major histocompatability complex (MHC) class I belong to the Ig superfamily of proteins and are present on the surface of virtually all nucleated cells. They are polymorphic transmembrane glycoproteins that function as receptors that present foreign antigen to cytotoxic T cells for destruction (Bjorkman et al., 1987). Ig binding of proteins displayed on the surface of Gram-positive bacteria is widely known. Staphylococcal protein A and Streptococcal Fc receptors bind Ig molecules but show little structural similarity between them (Navarre and Schneewind, 1999). *E. coli* produce fibrous surface proteins called curli that bind fibronectin and other matrix proteins and also interact with MHC Class I molecules (Olsen et al., 1998). The binding site for fibronectin is thought to partially overlap or be in close proximity to the MHC binding site on curli proteins (Olsen et al., 1998).

Precedence for fibronectin-binding proteins as an integral component of virulence is well documented for a number of bacterial pathogens including many Gram-positive bacteria (Navarre and Schneewind, 1999). These include *Streptococcal pyogenes* (M1 protein)(Cue et al., 2001), *Staphylococcus caprae* (Allignet et al., 2001), *Listeria monocytogenes* (Gilot et al., 1999), and *Mycobacterium avium* (Secott et al., 2001). Many

Gram-positive bacteria do not express structures such as pili, but utilize cell wall anchored surface proteins to adhere to extracellular matrices (Navarre and Schneewind, 1999). These proteins called microbial surface components recognizing adhesive matrix molecules, or MSCRAMMs bind to a variety of serum proteins including fibronectin (Joh et al., 1998, Navarre and Schneewind, 1999).

We have shown, that the peptide(s) involved in adherence and virulence in *Pasteuria* are MHC Class I like themselves, versus being receptors for MHC or other common cellular matrix molecules as described for a variety of other bacterial pathogens. One can merely speculate based on the lack of precedence for this interaction as to the origin of these gene products. The obligate nature of the interaction between the bacteria and its host and the observance of more than 100 genera of phytopathogenic nematodes having been described as having a *Pasteuria* pathogen suggests an ancient and highly integrated evolutionary development between the two organisms. Two potential theories emerge. One possibility is that owing to its growth and development within the living host, the bacteria acquires the immune machinery from the nematode and incorporates it onto itself as means to not only reside within the host but to have a template for future recognition. Another, and perhaps more likely scenario allows that as the result of a long evolutionary relationship with the host, *Pasteuria* acquired nematode genes by horizontal gene transfer. By displaying these gene products as means of recognition attachment the bacteria might avoid eliciting a deleterious host immune response and possibly signal some type of favorable response needed for entry and subsequent germination. Some pathogens rely on the action of the host immune response to successfully infect but without knowledge of the mechanisms by which *Pasteuria* spp. gain entry into and reside within the host, one can merely speculate. It would appear that *Pasteuria* serves no useful purpose to the nematode but this might not have been the case early in evolutionary history. It is possible that *Pasteuria* initially emerged as a symbiont, providing some advantage to the nematode, and was able to procure these genes only later to emerge as a pathogen. These questions will likely remain unanswered until the *Pasteuria* genome is sequenced. The finding of putative Eukaryotic genes of immune system origin displayed on the spore coat of a bacterial pathogen as a component of adhesion and virulence is a novel finding and requires further exploration.

CHAPTER 4
DETECTION OF *PASTEURIA PENETRANS* P20 INFECTED *MELOIDOGYNE ARENARIA* RACE 1 BY POLYMERASE CHAIN REACTION (PCR)

Introduction

An enzyme-linked immunosorbant assay (ELISA) using an anti-*Pasteuria* monoclonal antibody (MAb 2A41D10) has been demonstrated for detection of *Pasteuria penetrans* spores in soil and harvested root material. *Pasteuria*, in a manner consistent with other Gram-positive spore-forming organisms, undergoes vegetative propagation and growth prior to sporulation. In the infection process, soil-laden *Pasteuria* spores attach to the cuticle of an infective juvenile *Meloidogyne* (J2) as it migrates through the soil. Soon after the J2 enters a host plant and begins feeding, individual *Pasteuria* endospores, by as yet an unknown process, germinate and release a germ-tube or haustorium which penetrates the cuticle. Vegetative growth is characterized by development of thalli or mycelial-type microcolonies within the pseudocoelom of the host. Colonization of the haemolymph or pseudocoelomic fluid is extensive (Starr and Sayre, 1988). The onset of sporulation provides for morphological changes including the formation of tetrad followed by diad branched structures. Septa form at the apex of the diad and the structure is divided into two compartments, forming a pre-endospore which develops through a series of transformations similar to *Bacillus* sp. into a mature endospore (Chen et al., 1997; Ebert et al., 1996; Starr and Sayre, 1988). The epitope a product of sporulation (Brito et al., 1998, Brito et al., 1999) and constitutes a moiety associated with spore envelope as a component of virulence (Charnecki, 1997, Charnecki et al., 1998)

Since MAb 2A41D10 recognizes an epitope that arises as a product of sporulation, it cannot be used to detect vegetative and developing cells, thus reducing the capacity of the antibody based method for quantification of *Pasteuria in vivo*. Additionally, *Pasteuria* spp. have a finite but as yet incompletely defined host preferences and several different species and biotypes have been reported associated with different plant parasitic nematodes. Quantification of *P. penetrans* endospores in soil by immunoassay merely suggests the potential for nematode suppression but in itself is not indicative of host infection. The only sure evidence of infection is by detection of *Pasteuria* in nematode cadavers.

An obvious complement to immunodetection is a nucleic acid-based system for detection using PCR. This approach has inherent advantages and disadvantages, not withstanding that until recently the only known gene sequence for *Pasteuria* was for the 16s rRNA gene. *Pasteuria penetrans* spores were found to be quite recalcitrant to DNA extraction, with only 1 to 10% was released following treatment using a variety of chemical and physical methods. This discouraged development of a PCR approach for detection of *Pasteuria* in soil. However, vegetative and pre-sporulated cells present within the nematode host provide an available source of DNA, which can be readily released by traditional means.

Three gene specific oligomer pairs developed from *Pasteuria penetrans* P20 genes recently sequenced in this lab, *spoIIAB*, *sigE* and *atpF*, were used with real-time

PCR to amplify *Pasteuria* DNA in pre-sporulated cells obtained from infected *Meloidogyne arenaria* race 1. Fluorescent *in situ* hybridization (FISH) using a *sigE* gene probe shows hybridization of this sequence to *P. penetrans* cells in various stages of development. A robust nucleic acid based approach using *Pasteuria* specific primers and real time PCR is presented which is shown to distinguish infected versus un-infected *Meloidogyne* sp. residing *in vivo* within root tissue.

Materials and Methods

Meloidogyne arenaria cultivation for *Pasteuria penetrans* vegetative DNA production

Pasteuria penetrans isolate P-20 (Oostendorp et al., 1991) originated from *M. arenaria* (Neal) Chitwood race1, from Levy County, Fl., was grown on tomato (*Lycopersicon esculentum* Mill. cv. Rutgers) in greenhouses. P-20 spores were attached to the cuticles of 1 to 5 day-old *Meloidogyne* J2 via centrifugation at 1000 RPM for 5 min (Chen et al., 1996). Infected J2 were transferred via pipet to steam-pasteurized soil in 6 inch clay pots containing Rutgers tomato. Each plant was inoculated with approximately 10,000 infected *Meloidogyne* J2 in approximately 15 ml of dH_2O by adding the suspension to the topsoil using a bulb pipet. The plants were cultivated in an environmentally controlled growth room and exposed to 16 hours of light and 8 hours of dark and an average temperature of approximately 26 °C. Root systems were observed for the presence of gall formation and at 14 days the plants were harvested. Roots were harvested and washed free of debris and placed in 1 800 ml beaker containing 10% pectinase (Pomaliq, Gist-Brocades), 50 mM NaAc, 0.1% $CaCl_2$ at approximately a 50:50 (v:v) ratio. The roots were digested for 1 day on an orbital shaker table (100 RPM) to free spore-filled female *Meloidogyne* from the root material. Females were collected by decanting onto a 600 µm pore sieve nested on a 150 µm pore sieve under hard-water spray. Spore-filled females, identified by a translucent cuticle, were hand-picked by pipet with the aid of a dissecting microscope.

Pre-treatment of infected *Meloidogyne arenaria* to remove eukaryotic DNA

Approximately 300 *Pasteuria penetrans* P20 infected 14 day-old *Meloidogyne arenaria* females were washed in dH$_2$O and suspended in 1 ml of 10 mM Tris-HCl pH 8.0 containing 150 mM NaCl (T-NaCl). The suspension was transferred to a sterile glass mortar and disrupted by gentle grinding. The sample was centrifuged at 10,000 g for 5 min and the pellet was re-suspended in T-NaCl containing 5 mM MgCl$_2$. To this was added 50 µl of DNase (Promega) and 50 µl of RNase (Qiagen) and the sample was incubated at 37 °C for 40 min. Following centrifugation at 10,000 g for 5 min the pellet was re-suspended in T-NaCl containing 10 mM EDTA (TNE), centrifuged again, and re-suspended in cold TNE and stored at -20 °C.

Pasteuria penetrans DNA extraction

The DNase and RNase pre-treated cell preparation was re-suspended in 42.5 µl of TNE pH 8.0 and to this was added 10.0 µl of 100 mg/ml lysozyme. The sample was incubated for 2 hours at 37 °C with intermittent vortexing. Cell breakage was performed by subjecting the sample to three cycles of freezing and thawing using dry ice in ethanol followed by immersion in a 65 °C water bath. DNA was extracted using a QIAamp DNA Stool Mini Kit (Qiagen, Cat. no. 51504) and purified on a silica gel mini-spin column.

Crude DNA extraction of infected Meloidogyne within root galls

Tomato (*Lycopersicon esculentum* Mill. cv. Rutgers) root systems containing 14 day-old *Meloidogyne arenaria* race 1 infected with *Pasteuria penetrans* P20 were harvested and rinsed free of debris by hard water spray. Individual root galls (25 to30) were excised from the root-system and ground in a glass mortar and pestle. The sample was transferred to a 1.5 ml microfuge tube and centrifuged briefly to separate larger pieces of material. The supernate was transferred to a fresh tube and DNA extraction was performed as described previously using a QIAamp DNA Stool Mini Kit (Qiagen, Cat. no. 51504). Additionally, DNA was extracted using the Ultraclean Soil DNA kit (MO BIO Laboratories, Inc, Cat. no. 12800-50). Cell breakage was attained by boiling for 5 min at 95 °C followed by bead beating for 1 min using a dental amalgamator.

Real-time PCR

DNA was amplified in a 50 µl PCR reaction mixture containing 0.4 µl Amplitaq™ DNA polymerase, 5.0 µl of 10x Syber green, 4.0 µl of 12.5x $MgCl_2$ (Sybr® Green PCR Core reagents PE Biosystems), 5 µl of 10x dNTPs (Boehringer Mannheim, GmbH., Germany), 5.0 µl of DNA template, and a primer concentration of 5 µM. PCR was performed in a BIO-RAD iCycler iQ™ (Bio-Rad). Product formation was detected by the Syber green fluorophore (Life technologies Inc.) under the following thermal conditions, 50 cycles starting with a hot start then hold 30 s at 95 °C, annealing at 55 °C for 30 s, extending at 72 °C for 40 s and hold 1 min at 95 °C. Primer pairs used for *P. penetrans* were *sigE*–F, 5'-CCACGTATGCTTCGAGATGTA, *sigE*-R, 5'-CCCCCCTCCCAAACCAAAAC, *spoIIAB*-F, 5'-TCGTGTTTCTGTGGAGAT, *spoIIAB*-R, 5'-CGCAACGGCTACATTCCT, and *atpF*-F, 5'-GGTGTCGAAAATGATGGA, *atpF*-R, 5'-CAACCTCCGACTTAGCAG. Other primer pairs used included *Meloidogyne arenaria* specific rRNA primers, rRNA-F, 5'

CGGCTTCTGACGTGCAAATCGA, rRNA-R, 5'-TCCCACATCGCCAGTTCTGCT and *Lycopersicon esculentum* specific rRNA primers rRNA-F, 5'-GCAGCAGGCGCGCAAATTACC, rRNA-R, 5'-CCGATCCCGAAGGCCAACGTA and a rRNA combination primer to amplify *Meloidogyne arenaria* and *Lycopersicon esculentum*; rRNA-F, 5'-GCCGCGGTAATTCCAGCTCTGC and rRNA-R, 5'-CGGGCCGGGTGAGTTTCC. PCR products were detected by electrophoresis using a 2% agarose gel and stained in 0.1% ethidium bromide.

Fluorescence in-situ hybridization (FISH)

Meloidogyne arenaria race 1 cadavers containing *Pasteuria penetrans* P20 in early stages of development were removed from – 70 °C storage. Approximately 75 females were crushed using a plastic mortar and pestle and taken through a woven polyester 22 µm opening filter (Spectra/Mesh) in a 13 mm Swinnex disc filter (Millipore) to remove cuticle debris. The cells were washed in 50 % ethanol in sterile nanopure water and placed on ice in 50 % ethanol for 2 hours. Cover slips (12 mm, #1 thickness) were washed in 70% ethanol and rinsed with excess water. The cover slips were prepared for cell mounting by adding a solution of 1% Alcian blue in water and heating to almost boiling and incubating for 10 min in the heated water. The dye solution was poured off and the cover slips were rinsed in sterile water until becoming a uniform light blue in color. Cells were applied to cover slips and dried on a slide warmer for 5 min at 45 °C. The cells were dehydrated in a series of ethanol treatments consisting of 80% ethanol for 5 min followed by 96% ethanol for 5 min (Meier et al., 1999). Slides were air dried for 10 min and vacuum aerated to remove excess ethanol. Lysozyme (5 mg/ ml) was prepared in 100 mM Tris-HCl pH 8 0 containing 10 mM EDTA and added at a volume of 100 µl to the cover slips and incubated in a moist chamber at 37 °C for 40 min. The cover slips were rinsed with dH$_2$O and 3 µl of a 57.2 ng/µl 5' fluorescein conjugated DNA probe corresponding to a segment of the *Pasteuria penetrans* P20 *SigE* gene, F-CCCCCCTCCCAAACCAAAAC, was added to 20 µl of hybridization buffer (20 mm Tris-HCl pH 7.2, 0 9 M NaCl, 0 01% SDS, and 20% formamide) and applied to the cover slip Hybridization was performed at 46 °C for 1 5 hours in a moist chamber At 1.25 hours 80 µl of a 1 1000 dilution of DAPI stain was applied The cover slip was washed in 1.5 ml of dH$_2$O and air dried prior to mounting in Gel/Mount™ (Biomedia) (Amann et al , 1995) (Berchtold et al. 1999) Preparations were stored in the dark and visualized with a Nikon epifluorescent microscope fitted with a Episcopic fluorescence attachment that housed a 495 nm excitation filter under 40X fluorescent, or 100X DIC objectives with oil

Results

Real-time PCR of pretreated infected *Meloidogyne arenaria* race 1

Real-time PCR was performed on *Pasteuria* infected 14 day-old *M. arenaria* race 1 females that were isolated from tomato root tissue, ground and pre-treated with RNase and DNase to digest exogenous eukaroyotic DNA of nematode and plant origin. The following suspension was digested in lysozyme and followed by DNA extraction using the QIAamp DNA Stool Mini Kit (Qiagen). This DNA preparation was amplified by real-time PCR using *Pasteuria* specific *atpF*, *sigE* and *spoIIAB* primers. Product formation as evidenced by the Syber™ green fluorophore indicated that the three primers amplified DNA at cycle thresholds of 25, 26, and 28, respectively. Agarose gel electrophoresis showed PCR products consisted of one band of the predicted size for all three primers (Figure 37).

PCR of root galls containing infected and un-infected *Meloidogyne arenaria* race 1

Root galls excised from tomato plants (25 to 30 galls) containing 14 day-old *P. penetrans* infected *M. arenaria* race 1 were treated for DNA extraction using QIAamp DNA Stool Mini Kit (Qiagen) and the Ultraclean Soil DNA kit (MO BIO Laboratories, Inc). The crude DNA preparation was amplified by real-time PCR using *Pasteuria* specific *atpF*, *sigE* and *spoIIAB* primers. Product formation was observed using the Syber™ green 490 fluorophore which indicated that all primers amplified the template DNA (data not shown). Agarose gel electrophoresis of the PCR products demonstrated that amplified template DNA produced a single band of appropriate size for the three primer pairs (Figure 38). Template DNA acquired using the QIAamp stool Mini Kit is shown to provide a greater amount of PCR product than the Ultraclean Soil DNA kit based on cycle threshold values and band strength on the gel, but sample loss during processing likely contributed in large part to this result.

Real time PCR was performed on a crude DNA preparation of excised root galls (30 to 35 galls) containing uninfected female *M. arenaria* race 1 using *Pasteuria* primers *atpF*, *sigE* and *spoIIAB* to determine if these primers were specific to *Pasteuria* and capable of differentiating infected verses uninfected *Meloidogyne* in crude DNA extracts. The results indicated that no products were formed from the uninfected DNA control template and these primers appear strictly complimentary to *Pasteuria* DNA (Figures 39 and 40). Tomato (*Lycopersicon*) and *Meloidogyne* primers were applied to the template to validate the experimental conditions. Amplification of *Meloidogyne arenaria* rRNA and *Lycopersicon sp.* rRNA using species-specific primers is shown in Figure 40.

Fluorescence *in situ* hybridization (FISH)

Differential contrast microscopy (DIC) of material obtained from the body cavity of infected *Meloidogyne* female cadavers that was used for PCR experiments, shows *Pasteuria* in various stages of development (Figure 41). Here is observed a portion of a microcolony, as well as tetrads, diads and early stage endospores. These cells where fixed and used for fluorescent in-situ hybridization (FISH) using a fluorescein-conjugated *sigE* gene sequence, F-CCCCCCTCCCAAACCAAAAC as a probe. The results of *in situ* hybridization revealed the *sigE* probe bound strongly to *P. penetrans* (Figure 42a). Probe

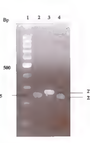


Figure 37 Agarose gel electrophoresis of PCR products following amplification of DNA extracts of *P. penetrans* P20 infected *Meloidogyne arenaria* race 1 females that were ground and pre-treated with RNase and DNase prior to bacterial DNA extraction. Lane 1: 100 kb marker; Lane 2 *Pasteuria penetrans spoIIAB* primer. Lane 3, *Pasteuria penetrans sigE* primer; Lane 4, *Pasteuria penetrans atpF* primer

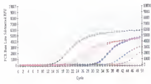


Figure 39 Real time PCR chromatogram of Syber™ green 490 fluorophore in response to product formation from DNA template obtained by extraction on root galls containing non infected *M arenaria* race 1 Primers used from left to right (lower to higher threshold cycle) *Lycopersicon* sp rRNA, *Meloidogyne arenaria* rRNA *Lycopersicon* and *M. arenaria* rRNA *P penetrans atpF P penetrans spoIIAB P penetrans sigE*

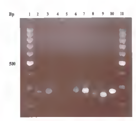


Figure 38. Agarose gel electrophoresis of PCR products from DNA extracted from ground tomato plant root galls containing *P. penetrans* P20 infected *Meloidogyne arenaria* race 1 and amplified using *P. penetrans* specific *sigE*, *atpF*, and *spoIIAB* primers. DNA was extracted using the Ultraclean Soil DNA kit (MO BIO Laboratories, Inc) (Lanes 3-6) and the QIAamp DNA Stool Mini Kit (Qiagen) (Lanes 7-10). Lane 1, 100 kb marker; Lanes 2 and 6, pre-treated DNA used as a control and amplified using the *P. penetrans sigE* primer pair. Lanes 3 and 7, *P. penetrans sigE* primer pair; Lanes 4 and 8, *P. penetrans spoIIAB* primer pair; Lanes 5 and 9 *P. penetrans atpF* primer pair; Lanes 6 and 10, 3x template DNA using *P. penetrans sigE* primer pair

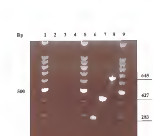


Figure 40 Agarose gel electrophoresis of PCR products of DNA extracted from ground tomato plant galls containing non-*Pasteuria* infected (uninfected) *Meloidogyne arenaria* race 1 using *Meloidogyne* rRNA, *Lycopersicon* rRNA and *P. penetrans* specific *sigE*, *atpF*, and *spoIIAB* primers. Lanes 1, 4 and 8, 100 kb marker; Lane 2, *P. penetrans sigE* primer pair; Lane 3, *P. penetrans spoIIAB* primer pair; Lane 4, *P. penetrans atpF* primer pair; Lane 6, *M. arenaria* rRNA primer pair; Lane 7, *Lycopersicon* rRNA primer pair; Lane 8, combination *M. arenaria* and *Lycopersicon* rRNA primer pair

concentration was highest as expected in the early vegetative stages of cellular development. A microcolony structure is shown to hybridize with the probe producing a distinct illumination of the mycelial microcolony (Figure 42b). The probe is also observed hybridizing to DNA in early stages of endospore development where it localizes in a polar region at the origin of what must become the spore cortex.

Phylogeny and the sigE gene

The *P. penetrans sigE* gene was converted to an amino acid sequence using the Translate tool software of ExPASy (http://us.expasy.org). σ^E sequences obtained from the SWISS-PROT and TrEMBL database (Bairoch and Apweiler, 2000) were aligned and analyzed using ClustalX and Treeview software. Phylogenetic bootstrap analysis placed *Pasteuria penetrans* in a clade with *Bacillus halodurans* and *B. subtilis* and *Clostridium perfringens* (Figure 43). *P. penetrans* σ^E displayed the greatest sequence homology to *Bacillus halodurans* with an 80.2 percent identity followed by *B. subtilis* with 78.3 percent, as determined by sequence alignment and percent identity matrix formulation using Peptool (Biotools software) (Figure 44).

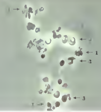


Figure 41 Differential interference contrast (DIC) micrograph (1000X) of the pseudocoelomic contents of *Meloidogyne arenaria* race 1 cadavers used for obtaining *Pasteuria penetrans* DNA. The micrograph reveals the presence of *P. penetrans* in various stages of cellular development including 1, branching thalli (microcolony), 2, tetrad, 3, diad and 4, early stage endospores.

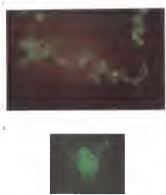


Figure 42. Epifluorescent micrograph of the fluorescent in situ hybridization (FISH) with the *P. penetrans sigE* gene probe illustrating positive hybridization to *P. penetrans* cells in various stages of development. Panel B shows intense hybridization of the probe to a microcolony illustrating the branched thalli characteristic of this early vegetative stage structure. Photos were taken using a Nikon microscope under epifluorescence using a 495 nm excitation filter at 1000X magnification under oil.

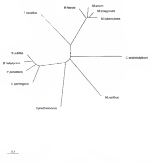
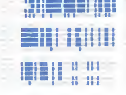


Figure 43 Bootstrap analysis using ClustalX and Treeview software of σ^E amino acid sequences showing the position of *P. penetrans* P20 within a clade containing *Bacillus* spp. and *Clostridium* sp..

A



B

	Pasteuria penetrans	*Bacillus halodurans*	*Bacillus subtilis*	*Clostridium perfringens*	*Clostridium acetobutylicum*
P. penetrans		80.2	78.3	70.8	16
B. halodurans	80.2		80.2	70.8	16.4
B. subtilis	78.3	80.2		71.9	15.8
C. perfringens	70.8	70.8	71.9		17.1
C. acetobutylicum	17.1	16.4	15.8	17.1	

Figure 44 Amino acid sequence alignment of σ^{h} using Peptools sofware (Biotools) showing consensus identity (A), and matrix percent identity (B) of *P. penetrans* with *Bacillus* and *Clostridium* spp

Discussion

PCR detection of *P. penetrans* P20 infection in *Meloidogyne* sp

Three *Pasteuria penetrans* genes, *sigE*, *atpF* and *spoIIAB* were recently obtained using a technique employing specific *Bacillus subtillis* primers (*atpF*) and *B. subtilis* degenerate primers (*sigE* and *spoIIAB*) for amplification and subsequent cloning of *Pasteuria* genomic DNA. PCR primers were developed from these sequences that successfully detected the bacterium within the *Meloidogyne* host at early stages of infection. These primers were analyzed by real-time PCR on DNA template obtained from RNase and DNase treated *Meloidogyne* isolated from root tissue as well as DNA obtained as a crude preparation from ground root galls containing infected *M. arenaria* race 1 in early stages of infection. Regardless of DNA source all three primers amplified template DNA to produce a single product of the predicted size. Crude DNA extracts of root galls containing *Pasteuria*-free (uninfected) *Meloidogyne arenaria* race 1 were not amplified by these primers. These results indicate that these three gene specific primers can be used in a rapid assay of crude DNA extract to differentiate infected from uninfected *Meloidogyne* residing within root systems.

However, the recalcitrance of mature endospores to DNA extraction may limit the ability to detect *Pasteuria* in aged root systems. Crop infection and re-infection by *Meloidogyne* continues throughout the active growing season. As roots elongate the tip exposes renewed areas for penetration by infective juvenile nematodes. When one views a root extension, the galls which are present furthest from the tip represent older infection sites containing more mature females compared to galls closer to the tip, thus representing in effect an infection continuum. Therefore, it would be advantageous to select galls nearer the root tip when selecting samples for *Pasteuria* detection by PCR. This approach would increase the likelihood of obtaining hosts that contain early stage bacterial development (if present) which is more amenable to DNA extraction, and thus detection by PCR.

A fluorescein-conjugated probe homologous to one of the *sigE* primer pairs succesfully hybridized to permeabilized *Pasteuria* cells in various stages of pre endospore development. This result demonstrated that the *sigE* primer was complimentary to *Pasteuria* DNA and thus PCR was amplifying a nucleic acid sequence present within this organism.

The *sigE* gene

The process of sporulation in *Bacillus subtilis* is a highly orchestrated and complicated process involving some 125 genes (Stragier and Losick, 1996). An important feature is the sequential activation of a variety of σ subunits of RNA polymerase which direct transcription of genes whose products drive the cell through morphological transitions resulting in the ultimate formation of an endospore (Kroos et al., 1999). Following replication and condensation of DNA to each of the two cell poles, sporulation of *Bacillus subtilis* involves the formation of a polar septum, which divides the mother cell from the developing endospore. The sigma E transcription factor encoded by the spoIIG operon is a cell-specific regulatory protein that directs gene expression in the mother cell (Fujita and Losick, 2002). It is produced initially as an inactive proprotein (pro-sigma E) and is proteolytically cleaved into an active form by a *spoIIGA* mediated processing event (Arcuri et al., 2000). Sigma E activity is confined to the mother cell where it directs transcription of several genes including *spoIIID* (Arcuri et al., 2000)

Evidence suggests that regulatory processes in the sporulation pathways of *Bacillus spp.* and *Clostridium spp.* are conserved (Arcuri et al., 2000). The *P. penetrans* σ^E sequence showed a high degree of homology and aligned in clade with *Bacillus halodurans*, *B. subtilis* and *Clostridium perfringens*. The data suggests a common origin of sporulation sigma factors for the *Bacillus*, *Clostridium* and *Pasteuria penetrans*. The diversity in the sequence of *sigE* is greater then for the 16S rDNA gene for the same group of organisms, allowing for the potential to develop a probe that will discriminate *P. penetrans* with different host specificities

CHAPTER 5
CONCLUSIONS

Pasteuria penetrans holds a myriad of challenges for study and development as a biocontrol agent of root-knot nematodes. Principally, our inability to culture the bacterium *in vitro* has limited the attainment of significant quantities of pure sample material with which to study and has prohibited classical bacteriological studies, particularly with regard to genetic analysis. As a parasite of a parasite (hyperparasite) its propagation relies on successful infection of two hosts and its sequestration there from. But despite *Pasteurias* unique, and what might be considered a clandestine niche, inroads are being made in understanding its role in the ecology of plant parasitic nematodes.

This body of work presents a novel extraction procedure to liberate bacterial endospore antigen in complex matrices and the development of a specific immunological probe, monoclonal antibody (MAb) 2A41D10, to detect and quantify virulent *Pasteuria penetrans* endospores in the environment. N-terminal amino acid sequences were obtained for peptides bearing the epitope recognized by the monoclonal antibody. The spore envelope epitope recognized by MAb 2A41D10 was shown to bind to the host, which allowed for partial elucidation of cuticle receptors on the nematode host. A nucleic acid-based detection system was also designed, using *P. penetrans* P20 gene specific primers and real time PCR, which allowed differentiation of infected versus un-infected *Meloidogyne arenaria* race 1 *in planta* in early stages of infection.

The primary objective involved developing an immuno-detection system for *P* *penetrans* and defining the nature of the epitope recognized by MAb 2A41D10. In order to assess the value of this probe it was necessary to determine if it was specific for *Pasteuria* and whether or not it detected an epitope involved in virulence. The monoclonal antibody was shown to be specific for *Pasteuria* sp. It did not recognize surface antigens on other Gram-positive bacterial endospores isolated from test soils and several American Type Culture Collection (ATCC) strains. It did not react with soil extracts that did not contain *Pasteuria*. It showed no cross-reactivity with *Meloidogyne* J2, *Meloidogyne* males, or free-living nematode cuticle protein. The monoclonal antibody binds *Pasteuria* spore coat extracts obtained from pure culture and from spore-laden soil in a dose dependent manner. This provided the basis for a quantitative ELISA of endospores in soil. Signature peptides were observed on immunoblots of spore antigen extracted from soil. Antibody detection of soil spore antigen extracts by enzyme-linked immunosorbant assay (ELISA) was independent of soil type for the three soils tested. MAb 2A41D10 bound spore antigen for all *P. penetrans* isolates tested.

Immunoblots of spore envelope extracts presented an antigenic ladder rather than a single molecular weight peptide. It appears that the MAb recognizes an epitope distributed on a variety of molecular weight peptides. Antigenic ladders of four biotypes (P20, P100, P120 and B4) (Oostendorp et al., 1990) all differed somewhat. However, variation in the profiles may not be distinct enough to be used for definitive qualitative identification. Comparison of the profiles does suggest that peptide distribution and variations in size, possibly a result of degree or type of glycosylation, may play a role in host preference. Davies et al., who found differences in the amount and nature of the proteins among spore populations with studies using a polyclonal antibody, suggest these differences may account for variation in host specificity, but this has yet to be proven (Davies et al. 1992). It is conceivable that the distribution of an adhesin epitope on multiple peptides would increase the probability of attachment to a dynamic host receptor. Additionally, the antigenic profiles of progeny from spores that were forcibly attached (by centrifugation) and propagated on four different *Meloidogyne* populations, including both preferred and non-preferred host species, were not significantly altered from parent populations. Decreased propagation was observed in instances where it was difficult to attach spores, but in no case did propagation fail entirely. Forcible attachment and subsequent propagation resulted in little apparent change to the immunogenic profile. To provide definitive results, this experiment should be repeated and several generations or passages evaluated. Antigenic gene shuffling occurs within many parasitic populations. An immunogenic profile merely represents an average of the population at a single point in time. The relative functional ambiguity regarding suitable hosts and the ability to propagate spores on non-preferred hosts follows previous research and emphasizes the difficulty understanding the dynamics of *Pasteuria* and nematode ecology. However, the observation that this monoclonal antibody recognizes a common epitope shared on a number of *Pasteuria* species suggests that a common epitope exists which is common and unique to the genus *Pasteuria*.

The results indicate that this monoclonal antibody detects an epitope that is either directly involved in attachment or comprises a component of a peptide involved in recognition and attachment to the host. Evidence for this comes as a result of several defining experiments. Principally, this MAb bound to spore envelope protein that bound to the surface of infective *Meloidogyne J2*. This was shown by ELISA (in a dose dependent manner), Far Western blotting, and immunofluorescent microscopy. *Pasteuria* biotype P20 spore extract bound to both *M. arenaria* race 1 and *M. incognita* race 3 cuticle extracts. N terminal amino acid sequence analysis of spore coat peptides bearing the MAb 2A41D10 epitope collectively had the greatest similarity to *Caenorhabditis elegans* proteins and displayed a motif with homology to major histocompatabity complex (MHC) Class I gene products. They bore distant homology generally, to Prokaryotes. They show no similarity to spore coat proteins of other Gram positive *Bacillus* spp., which are phylogenetically close relatives. This is an unprecedented finding that warrants further research.

Gene specific primers were developed from genomic DNA sequences recently obtained from *P. penetrans* P20. Real-time PCR using the *sigE*, *atpF*, and *spoIIAB* gene primer pairs allowed differentiation of infected versus un-infected *Meloidogyne arenaria* race 1 in crude DNA extracts of root-galls. A nucleic acid based detection system sensitive and specific for the detection of *Pasteuria* vegetative cellular development in the host will compensate for limitations in immuno-detection of pre-endospore cells. It can also be used to validate infection when spores are detected in the environment.

Pasteuria species are distributed worldwide amongst 116 genera and 323 species of free-living, predatory, plant-parasitic, and entomopathogenic nematodes (Chen and Dickson, 1998). There are likely many more *Pasteuria*-like parasites in the natural world that are yet to be discovered. Today, two phyla, the Arthropoda and Nematoda have described *Pasteuria* species as obligate parasites. The *Daphnia* spp. (water-flea) parasite, *P. ramosa*, is the type species for the genus (Ebert et al., 1996). Three plant parasitic nematode parasites have been described. *P. thornei* a parasite of the lesion nematode, *Pratylenchus* sp., *P. nishizawae* a parasite of the cyst nematode, *Heterodera* spp. and *Globodera* spp., and *P. penetrans* which infects *Meloidogyne* spp. (Sayre and Starr, 1989). At least one isolate, *P. penetrans* biotype B4 infects both *Pratylenchus* sp. and *Meloidogyne* spp. (Chen and Dickson, 1998). Recently, two new *Pasteuria* sp. have been described from the ring nematode *Criconemella* spp. and the sting nematode *Belonolaimus* spp. (Bekal et al., 2001) based on 16S rDNA sequence analysis.

Pasteuria species have historically been differentiated based upon host preference, life cycle, ultrastructure, and morphology (Sayre and Starr, 1989) which has led to considerable confusion regarding its taxonomy (Chen and Dickson, 1998). 16S rDNA sequencing has been used to phylogenetically place existing and newly described species (Anderson et al., 1999, Atibalentja et al., 2000). 16S rDNA analysis using various parsing methods finds *Pasteuria* rooted within the *Clostridium-Bacillus-Streptococcus* branch of the Gram-positive eubacteria and descended from the *Alicyclobacillus* group of the *Bacillaceae* (Atibalentja et al., 2000). Host preference between *P. penetrans* biotypes is not differentiated by 16S rDNA sequence analysis. *P. penetrans* biotypes P20 and P100 have virtually identical 16S rDNA sequences (Anderson et al., 1999). Emerging molecular phylogenetic markers include use of the *sigE* gene of endospore-forming Gram-positive bacteria. Sigma factors are transcriptional rRNA polymerase regulators produced by the mother cell that regulate sporulation. They are highly conserved within *Clostridium* spp. and *Bacillus* spp. (Arcuri et al., 2000). Our finding that *P. penetrans* *sigE* gene lies in clade with these two genera helps to establish the evolutionary relationship between *Pasteuria* and other Gram-positive spore-formers. The sporulation genes including *sigE* may as well provide a more discriminating means to speciate *Pasteuria* once gene sequences are obtained from other isolates.

Conflicting evidence exists for nematode phylogeny as a basis for host specificity. Some have suggested that *Meloidogyne* species could be differentiated based upon spore binding of distinct populations of *Pasteuria* (Oru and Ozawa, 1999). Others provide evidence that phylogeny is not a basis for host specificity (Stirling, 1985) (Davies et al., 2001), and this is at least partially explained by intra-generic cuticle heterogeneity (Davies and Redden, 1997). Spore attachment has been observed to vary 100-fold between *Meloidogyne* populations but evidence was not provided to support a complete lack attachment (Stirling, 1985). Studies here showed *P. penetrans* biotype P20 bound all *Meloidogyne* spp. tested (*M. incognita* race 1 and 3, *M. arenaria* race 1 and 2 and *M. javanica* race 1) but with different affinities at a 0.05 level of significance by single factor ANOVA analysis. Host preference deviated from the predicted. *M. arenaria* race 1 is the defined host for biotype P20 and the results indicated *M. arenaria* race 2 demonstrated the highest level of attachment. The capacity of spores to attach to the cuticle is not a direct indicator of host suitability because germination is required for successful infection (Sayre and Wergin, 1977). However, poor attachment may result in reduced infection because not all spores will successfully germinate. Unfortunately, many observations of intra- and inter generic spore attachment has not been followed up with propagation studies to verify infection (Chen and Dickson, 1998).

Herein lies the tale of the two moving targets. The ubiquity of *Pasteuria* in nematode ecology and its obligate parasitism has undoubtedly resulted in highly evolved mechanisms of "hide and seek" in regard to their "host and parasite" relationship that will be difficult to define and predict using taxonomy alone. Evidence for this problem fills the scientific literature over the past 20 years. Elucidation of the biochemical and molecular basis for recognition and attachment may provide the best approach for determining and classifying host preference as it relates to biocontrol.

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BIOGRAPHICAL SKETCH

Liesbeth M. Schmidt was born and raised in Kendall, Florida. She attended Palmetto Elementary, Jr. and Sr. High schools where she played the French horn in marching and symphonic band, and excelled as an athlete competing for the Florida State title in discus throwing and softball. She obtained a Bachelors of Science Degree in Agriculture from the University of Wyoming in Laramie, Wyoming in 1984. While there she worked summers for the U.S. Forest Service and also for the Union Pacific Railroad in a wood treatment yard. Upon graduation she returned home and took an entry-level position with the Suwannee River Water Management District (SRWMD) in Live Oak, FL and became a District Hydrologist. She worked for the Alachua County Office of Environmental Protection in Gainesville, FL as an Environmental Engineer/Project Manager overseeing establishment and implementation of the ambient groundwater monitoring network before beginning graduate studies at the University of Florida. Her principal focus to this point involved groundwater chemistry and hydrology. She began graduate studies at the University of Florida in the Department of Environmental Engineering in 1992. She received a Master of Science degree in 1996. Her graduate study interest was microbial degradation of low aqueous phase concentration pentachlorophenol in groundwater. Her research was funded by a private hazardous waste clean-up facility where she worked as a technical assistant while conducting her research. In mid 1997 she began her Doctoral research at the Department of Microbiology and Cell

Science under the mentor of Dr James F Preston. Her she developed an immunological approach to detect and quantify *Pasteuria* bacterial endospores in complex matrices and to elucidate the components involved in virulence of the spore and its receptors on the nematode host. She is a Board of Directors member of the Columbia County Cattlemen's Association and is a member of Gamma Sigma Delta. She owns and operates a cow/calf ranching operation in Columbia County, FL. She plans to continue her academic study as a postdoctoral research fellow

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy

James F Preston, III, Chair
Professor of Microbiology and Cell Science

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Henry C Aldrich
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Professor of Microbiology and Cell Science

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Donald W. Dickson
Professor of Entomology and Nematology

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Robert McSorley
Professor of Entomology and Nematology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy

Madeline E Rasche
Madeline E Rasche
Assistant Professor of Microbiology and
Cell Science

This dissertation was submitted to the Graduate Faculty of the College of Agriculture and to the Graduate School and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy

August, 2002

E. Jane
Dean, College of Agriculture

Dean, Graduate School